Predictors of Disease Extension and Progression in Patients with Granulomatosis with Polyangiitis (GPA)

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Thesis Declaration

I, Hazlita Mohd.Isa, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Granulomatosis with Polyangiitis (GPA) is a granulomatous inflammatory disease. It is generally described as a systemic disorder which can present with a localized presentation like the orbit. Orbital GPA can be the initial manifestation of GPA where over time the disease may progress and become severe, involving vital organs. This study aimed to look for biomarkers in orbital GPA biopsies that could indicate diagnosis and be a predictor for the progression of the disease.

To identify GPA patients, retrospective examination of patients' medical records, who had undergone orbital biopsies for orbital inflammatory disease (OID), over a 21 year period, was performed. Long term outcomes of these patients were studied. Further subjective and objective histology analyses were done on haemotoxylin and eosin (H&E) tissue preparations. Comparison of cellular activity in biopsies of GPA and other OID were performed. Further T cells, B cells and macrophage phenotypes and their cytokines, were investigated with immunohistochemistry (IHC). IHC cell count comparisons were performed between GPA, sarcoidosis and idiopathic inflammatory orbital diseases (IIOD) biopsies.

Results showed that in patients who presented with orbital GPA with no systemic manifestations, the disease remained localised and did not progress to systemic form, over time. H&E tissue biopsies examination showed that GPA tissues had a higher cellular activity compared to OIDs. Vasculitis and necrosis were found to be independently associated with the diagnosis of orbital GPA but these features were unreliable for diagnosis as a number of the biopsies did not exhibit these features. In immunohistochemistry staining, T cells, B cells and macrophage subtypes counts were comparable between GPA, sarcoidosis and IIOD. Nonetheless cytokines IL-17, IL-23 and

BAFF-receptor (BAFF-R), were found significantly more in GPA compared to sarcoidosis and IIOD. This suggests that these cytokines possibly have a role in the pathogenesis of GPA and may have diagnostic value.

Summary

Introduction

GPA is a granulomatous inflammatory disease, generally regarded as a systemic disorder and could potentially be life threatening. Although the disease is more commonly described in larger major organs (systemic GPA), it is also recognised to occur in less life threatening organs such as the eye and nose (localised GPA). Localised GPA is generally thought to represent the earlier stage of the systemic manifestation of the disease. Localized GPA is difficult to diagnose, as early symptoms are often similar to other inflammatory conditions and investigations are usually negative. Therefore there is a potential for a diagnostic delay to occur where the disease often only becomes apparent after the disease has progressed; causing irreversible organ damage or disease spread to major organs. The aims of this study were therefore to look for potential biomarkers in the orbital biopsies, for the diagnosis of GPA as well as to predict disease progression. The study was divided into several parts.

Long term outcome of orbital GPA

In this first section of the study, the main aim was to identify patients who were diagnosed with orbital GPA who progressed into the systemic form of the disease. We also observed the patient's demographic pattern, ANCA status, treatment modalities that were advocated and general long term outcomes. Our main findings were that, all of our patients diagnosed with orbital GPA, who had no associated systemic manifestations at presentation, did not progress to the systemic form of the disease over time. Patients, who

were characterised as systemic GPA, had systemic involvement prior to their orbital presentations.

ANCA was positive in 60% of our patients however only about 40% had a positive serological ANCA investigation at the time of presentation. Patients with negative ANCA investigations can become positive over time thus repeated testing should be done in these patients particularly in cases with recurrent and severe attacks.

Histology comparison between GPA and other OIDs

Histology plays an important role in the diagnosis of GPA. Classical pathological features seen in GPA tissue biopsies stained with H&E include granulomatous inflammation, vasculitis of small to medium size vessels and tissue necrosis. These features are however not always apparent in orbital GPA tissues. This study was to compare tissue changes and cellular activity in GPA to OIDs to determine if there are factors that could differentiate them histologically.

We were not successful in identifying a biomarker in H&E tissue biopsies that could be used as a biomarker for the diagnosis of orbital GPA. Yet we found that the cellular activities in GPA orbital biopsies were higher compared to the combined cellular activities of OIDs. This result reflects the more severe clinical course of orbital GPA compared to OIDs. In addition, necrosis and vasculitis were independent factors associated with the diagnosis of orbital GPA, consistent with the pathological picture of GPA in other organs. Nevertheless, these features were not consistently seen in all tissue biopsies of orbital GPA in our samples, thus cannot be a reliable feature for the diagnosis of orbital GPA. Granuloma was found to be inversely associated with the diagnosis of orbital GPA thus the presence of this feature in orbital biopsies suggests that the diagnosis of GPA is unlikely.

T cells in GPA

The involvement of T cells as well as its various subsets in GPA is well documented. CD4 and CD8 have been demonstrated in GPA affected tissues such as the kidneys and CD134 T memory cells has been shown to be present in lungs affected by GPA as well. Th17 and its IL-17 cytokine in particular, has been the centre of attention in the development of many autoimmune inflammatory diseases including GPA. In this section of the study we wanted to investigate the presence of T cells and its subsets, as well as cytokine IL-17, in orbital GPA as well as compare them to T cell presence in orbital sarcoidosis and IIOD. This was done by immunohistochemistry (IHC).

We found that CD3, CD4, CD8, CD134 and IL-17 were all present in all three orbital diseases. CD3, CD4, CD8 and CD134 counts were similar between GPA, sarcoidosis and IIOD. There was also no cell type preponderance noted; unlike seen in kidneys affected by GPA where CD8 predominates. More important, IL-17 was found to be markedly higher in GPA compared to both sarcoidosis and IIOD. Thus despite appearing to be involved in all three orbital diseases, the role of IL-17 seems to be more important in orbital GPA rather than other OIDs.

On another note, in our sarcoidosis tissues, the CD4 and CD8 counts were similar where the CD4/CD8 ratio was close to one. This did not concur with findings in other organs affected with sarcoidosis. Bronchoalveolar lavage fluid investigations in patients with pulmonary sarcoidosis showed that a CD4/CD8 ratio of two or more, were indicative the disease.

CD134 previously demonstrated in endonasal biopsies in GPA is a member of the TNFR-superfamily of receptors. It is suggested that the level of CD134 is associated with GPA disease activity. We did not find a significant difference in the presence of CD134 within GPA, sarcoidosis and IIOD indicating that this T cell subtype may be significant in

inflammation in general and not specific in GPA development. To our knowledge this is the first time CD134 is observed in sarcoidosis and IIOD.

B cells in orbital GPA

B cells are also implicated in the pathogenesis of GPA, particularly after Rituximab; a chimeric monoclonal antibody against the protein CD20, has been shown to be effective in the treatment of this disease. BAFF is a cytokine that helps maintain and prolong the survival of B cells. It binds with the receptor BAFF-R on B cells. The BAFF/BAFF-R interaction has been shown to be present in endonasal biopsies of patients with GPA and is said to be important in the disease development. In this section, we investigated the presence of B cells (CD20) and BAFF cytokines as well as BAFF-R, in orbital GPA by IHC, and compared them to B cell presence in orbital sarcoidosis and IIOD.

We were unsuccessful in staining for BAFF in our sample tissues however were able to demonstrate presence of CD20 and BAFF-R in all three orbital diseases. CD20 counts were comparable between the three diseases indicating that the role of B cells was similar in all three diseases. However BAFF-R expression were significantly higher in GPA compared to sarcoidosis and IIOD, signifying a possible significant positive influence of BAFF in GPA disease development. Thus while B cells were equally important in all three orbital diseases, B cells role in the inflammatory process in GPA may be sustained longer due to the prolonged survival time and maintenance of B cells in tissues by BAFF.

Macrophages in GPA

Despite GPA being described as a granulomatous disease (an inflammation predominated by macrophages), investigations on macrophage and its various phenotypes, in the disease development, are surprisingly scarce. CD68 has been described to be present in tissues affected by GPA such as in the kidneys. Other M2 (alternative activated macrophages) have not been identified in GPA. IL-23 cytokine has been shown to have a role in the pathogenesis of AAV including GPA but its presence in orbital GPA has not been shown. In this part of the study, we performed IHC to look at the presence of macrophage subtypes and cytokines, which included CD68, CD204, CD163 in addition to cytokines IL-23 and AIF1, in orbital GPA, sarcoidosis, IIOD and compared the presence of these immune cells between GPA and IIOD biopsies plus GPA and sarcoidosis biopsies.

We were unable to get a conclusive stain for CD163, CD204 and AIF1. CD68 and IL-23 were found to be present in all biopsies from all three orbital diseases. CD68 was found to be significantly higher in GPA when compared to IIOD but CD68 counts were comparable between orbital GPA and sarcoidosis. The granulomatous nature of GPA explains the difference in the presence of these macrophages with IIOD and its similarity to sarcoidosis which is also a granulomatous disease. IL-23 in particular was shown to be significantly higher in the biopsies of GPA when compared with both IIOD and sarcoidosis. This cytokine appears to play an important role in the disease development of GPA. The function of IL-23 is to promote production of IL-17, a proinflammatory cytokine via Th17. As IL-17 was found to be high in orbital GPA too, the IL-23/IL-17 pathway may be the key player in the pathogenesis of GPA.

Conclusion

Our study shows that orbital GPA with no systemic presentation may remain solely in the orbit and may not have systemic involvement. Cellular activity in orbital GPA is higher compared to OIDs. This was seen in both subjective and objective immune cell and tissue change counts. This result reflects the disease severity compared to OIDs. Tissue necrosis and vasculitis were features independently associated with the diagnosis of GPA in the orbits, similar to other organs affected by GPA. However these features were not consistently found in all orbital GPA tissues thus may not serve as a good indicator for disease diagnosis. The main difference found in the immune cells between orbital GPA, sarcoidosis and IIOD were not in the main cell subtypes, but in the cytokines production. IL-17, IL-23 and BAFF-R were found significantly more in GPA compared to sarcoidosis and IIOD. Therefore it may be suggested that the presence of these cytokines in orbital biopsies together with positive clinical correlations may be used as a diagnostic marker for the diagnosis of orbital GPA.

Publications and Poster Presentations

Publication arising during the period of the thesis

1. Ocular manifestations of Wegener's granulomatosis. Hazlita Isa; Susan Lightman; Charles D Pusey; Simon RJ Taylor. Expert Review of Ophthalmology. 2011; 6(5):541-555.

2. Histopathological features predictive of a clinical diagnosis of ophthalmic granulomatosis with polyangiitis (GPA). Hazlita Isa; Sue Lightman; Philip J. Luthert; Geoffrey E. Rose; David H. Verity; Simon R.J. Taylor. International Journal of Clinical and Experimental Pathology. 2012; 5(7):684-689.

3. Diagnostic value of IL-17, IL-23 and BAFF-R in localised granulomatosis with polyangiitis. Hazlita Isa, Philip J Luthert, Geoffrey E Rose, David H Verity, Charles Pusey, Oren Tomkins-Netzer, NorshamsiahMd Din, Simon RJ Taylor, Sue Lightman (submission in progress)

4. Clinical and Imaging Features Predictive of Orbital Granulomatosis with Polyangiitis and the Risk of Systemic Involvement. Tan LT, Davagnanam I, Isa H, Taylor SR, Rose GE, Verity DH, Pusey CD, Lightman S. Ophthalmology. 2014 Feb 20. pii: S0161-6420(13)01175-5. doi: 10.1016/j.ophtha.2013.12.003. [Epub ahead of print]

5. Prevalenceandcausesofphthisisbulbiinpatientswithocularinflammation. Letter to Editor. *ActaOphthalmologica*2011. Lee T. Tan, Hazlita Isa, Sue Lightman, Simon RJ Taylor.

Conference presentations (Poster)

1. Royal Congress of Ophthalmology (RCO), Liverpool, UK. 25th – 27th May 2010. Histopathological Features of Orbital Biopsies Associated with the Clinical Diagnosis of Wegener's Granulomatosis. H Isa, L T Tan, S R J Taylor, P J Luthert, S L Lightman. UCL Institute of Ophthalmology, UK

2. World Ophthalmology Congress (WOC) 2010. Berlin, Germany. 5-9 June 2010. Histopathological features of Orbital Biopsies associated with the Diagnosis of Wegener's Granulomatosis. Hazlita Isa; LT Tan, Norshamsiah MD, SRJ Taylor, PJ Luthert, D Verity, GE Rose, Sue Lightman.

3. ARVO - The Association for Research in Vision and Ophthalmology. Fort Lauderdale, Florida, USA. 1-5 May 2011. Analysis of Cellular Infiltration in Orbital Biopsies of GPA and Non GPA patients). Hazlita Isa; Sue Lightman; Philip J. Luthert; Geoffrey E. Rose; David H. Verity; Simon R.J. Taylor.

4. World Ophthalmology Congress (WOC) 2010. Tokyo, Japan. 2-6 April 2014. Diagnostic value of IL-17, IL-23 and BAFF-R in localised granulomatosis with polyangiitis. Hazlita Isa, Philip J Luthert, Geoffrey E Rose, David H Verity, Charles Pusey, Oren Tomkins-Netzer, NorshamsiahMd Din, Simon RJ Taylor, Sue Lightman

5. British Oculoplastic Surgery Society Meeting (BOPSS), Bristol. 19th – 21st June 2013. Clinical and imaging features predictive of GPA in orbital inflammation. Tan LT, Davagnanam I, Isa H, Taylor S, Rose GE, Verity DH, Pusey CD, Lightman S.

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List of abbreviations

AAV	-	ANCA associated vasculitides
ACE	-	Angiotensin converting enzyme
ACR	-	American College of Rheumatology
ANCA	-	Anti-neutrophil cytoplasmic antibodies
APC	-	Antigen presenting cells
APRIL	-	a proliferation inducing ligand
AZA	-	Azathioprine
BAFF	-	B-cell activating factor
BAFF-R	-	B-cell activating factor receptor (BR3)
BALF	-	Bronchoalveolar lavage fluid
BCMA	-	B-cell maturation antigen
BCR	-	B cell receptor
c-ANCA	-	Cytoplasmic Anti-neutrophil cytoplasmic antibodies
CD	-	Cluster of differentiation number
CHCC	-	International Consensus Conference at Chapel Hill
CS	-	Corticosteroids
CSS	-	Churg-Strauss syndrome
CT	-	Computerised Tomography
CYC	-	Cyclophosphamide
DNA	-	Deoxyribonucleic acid
DPX	-	DPX mountant
Elisa	-	Enzyme-linked immunosorbent assay
EULAR	-	The European League Against Rheumatism
EULAR/PReS	-	Paediatric Rheumatology European Study
EUVAS	-	European Vasculitis Study Group
FDG PET/CT	-	[18F]-fluorodeoxyglucose Positron emission tomography /Computerised Tomography

GM-CSF	-	Granulocyte-macrophage colony-stimulating factor
GPA	-	Granulomatosis with polyangiitis
H&E	-	Haematoxylin and Eosin
IgE	-	Immunoglobulin E
IgG	-	Immunoglobulin G
IgG4	-	Immunoglobulin G 4
IHC	-	Immunohistochemistry
IIOD	-	Idiopathic inflammatory orbital disease
IL-3	-	Interleukin 3
IL-4	-	Interleukin 4
IL-5	-	Interleukin 5
IL-6	-	Interleukin 6
IL-10	-	Interleukin 10
IL-12	-	Interleukin 12
IL-13	-	Interleukin 13
IL-17	-	Interleukin 17
IL-17A	-	Interleukin 17A
IL-21	-	Interleukin 21
IL-22	-	Interleukin 22
IL-23	-	Interleukin 23
IFN- γ	-	Interferon gamma
LAMP2	-	Lysosomal membrane protein 2
MEH	-	Moorfields Eye Hospital
MHC	-	Major histocompatibility complex
MPA	-	Microscopic polyangiitis
MPO	-	Myeloperoxidase
MRI	-	Magnetic resonance imaging
MTX	-	Methotrexate
NK cells	-	Natural killer cells

NSAID	-	Non-steroidal anti-inflammatory drugs
OID	-	Orbital inflammatory diseases
PAN	-	Polyarteritis nodosa
p-ANCA	-	Perinuclear antineutrophil cytoplasmic antibodies
PE	-	Pulmonary embolism
PR3	-	Proteinase 3
PUK	-	Peripheral ulcerative keratitis
RA	-	Rheumatoid arthritis
RPGN	-	Rapidly progressive glomerulonephritis
RTX	-	Rituximab
SLE	-	Systemic Lupus Erythematosus
TACI	-	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TED	-	Thyroid Eye Disease
TGFβ	-	Transforming growth factor beta
Th1 T cells	-	T Helper 1 T cells
Th1 T cells7	-	T Helper 17
Th2 T cells	-	T Helper 2 T cells
Thαβ	-	T Helper Alpha Beta
TLR	-	Toll like receptor
TLR2	-	Toll like receptor 2
TLR9	-	Toll like receptor 9
TNF	-	Tumor necrosis factor
TNF-α	-	Tumor necrosis factor alpha
Treg	-	T regulatory cells
UCL	-	University College of London
WG	-	Wegener's granulomatosis
WGET	-	Wegener's granulomatosis Eternecept Trial

1 Chapter 1: Introduction

1.1 Perspective

Orbital granulomatosis with polyangiitis (GPA) is an orbital inflammatory disease that poses a diagnostic challenge. Generally, the disease has similar clinical presentation as other orbital inflammatory diseases, such as idiopathic inflammatory orbital diseases (IIOD) and sarcoidosis. In addition, investigations from serology blood test with anti-neutrophil cytoplasm antibody (ANCA) which is strongly associated with the disease often do not yield a positive result. Localised orbital GPA may progress to involve other major organs like the kidneys, where the disease is then termed as systemic GPA, and can be potentially life threatening if appropriate and adequate treatment is not advocated early. On the other hand, treatment for GPA often involves the use of strong immunosuppressants that carry severe side effects themselves, thus they must be used with caution and only when necessary. Therefore, it is indeed important for us to be able to make a quick and correct diagnosis. Histology biopsy plays an important role in the diagnosis of orbital GPA. However, the evaluation usually involves looking for features consistent with GPA that has been described before in other larger organs affected by GPA, such as the lungs and kidneys. Again, these features may not always be present in orbital samples because tissue biopsies from the orbit are usually small and limited. Hence, it would certainly be of benefit if a cellular biomarker could be identified in tissue biopsies to diagnose orbital GPA and possibly GPA in general, and potentially also be used to predict the disease progression and outcome. In order for us to explore further, it would indeed be helpful to first understand GPA in general, particularly the ocular manifestations of the disease.

1.2 Introduction

Granulomatosis with polyangiitis (GPA) (previously known as Wegener's granulomatosis (WG)) was initially described by Heintz Klinger, a medical student, in 1931. The disease was described to be a variant of another vasculitic disorder; polyarteritis nodosa (PAN). However, in 1934 and 1939, Frederick Wegener; a German pathologist, further defined the disease in greater detail and showed that the disease can be clearly distinguished from other inflammatory diseases such as PAN and is actually a distinct inflammatory disease(Rasmussen et al., 1990)(Langford, 2001).Since the discovery by Frederick Wegener, the disease then later adopted his name as Wegener's granulomatosis. Recently however, The Boards of Directors of the American College of Rheumatology, the American Society of Nephrology, and the European League Against Rheumatism(Falk et al., 2011)recommended the term granulomatosis with polyangiitis (GPA) as an alternative to Wegener's granulomatosis, to better describe the pathology and to avoid eponyms.

GPA is pathologically described as a multisystem granulomatous necrotising inflammation which is associated with pauci-immune vasculitis of small- to medium-sized blood vessels and is presumed to be of autoimmune origin (Holle et al., 2010a). GPA can affect any organ system but the disease is seen to have a pre-disposition to affect the upper and lower respiratory tract system and the kidneys. Together with other known vasculitic diseases which include Churg Strauss syndrome (CSS), microscopic polyarteritis (MPA) and renal limited vasculitis, GPA has been shown to have a strong association with a specific antibody called anti-neutrophil cytoplasm antibody (ANCA). Collectively, these groups of disorders are known as the ANCA-associated vasculitides (AAV)(Isa et al., 2012). Despite this strong association with ANCA, a positive ANCA serology is not always established in all cases although the absence of ANCA does not preclude diagnosis either.

In the eye, GPA may involve any ocular structure, and ocular manifestations of the disease vary. It has been shown that up to 60% of patients with GPA manifest ocular involvement. In addition, ocular signs can be the initial presentation of the disease (Pakrou et al., 2006).

1.3 Epidemiology

The overall incidence of GPA is estimated to be approximately 4 to 8.8 cases per million people, although this varies depending on the locality. In the UK, the annual incidence of GPA is reported to be towards the higher end at 8.4 cases per million. (Watts et al., 2000) GPA is said to occur more commonly among Caucasians(Hoffman et al., 1992). Based on epidemiological studies, it is suggested that the prevalence of the disease appears to have an apparent latitude dependent predisposition, with a decreasing north-south gradient in the northern hemisphere and a reciprocally increasing south-north gradient in the southern hemisphere (Koldingsnes and Nossent, 2000)(O'Donnell et al., 2007)(Gibson et al., 2006)(Hissaria et al., 2008). The reasons for this remain unclear. One study suggested that this distribution may be influenced by ethnicity rather than the geographical location (Faurschou et al., 2013). Cases of GPA have been reported throughout other parts of the world such as in Japan, India and China(Kobayashi et al., 2010)(Kumar et al., 2001)(Chen et al., 2008)(Singer et al., 1990), and in Taiwan, the annual incidence of GPA is said to be around 0.37 per million patients per year (Wu et al., 2014).

The incidence of GPA is seen to peak in the sixth decade of life and there is no apparent gender predilection. GPA has generally been considered as a relatively rare disease but recent studies have shown otherwise. The prevalence of GPA has been shown to have tripled over a 15 year period(Koldingsnes and Nossent, 2000)(Gibson et al., 2006) with a concurrent rise in incidence seen as well(Watts et al., 2000)(Takala et al., 2008). The

disease is also seen to be affecting patients of a younger age (Watts et al., 2009)(Koldingsnes and Nossent, 2000)(Hissaria et al., 2008)(Takala et al., 2010).

1.4 Systemic (General) versus Localised (Limited) GPA

GPA has a wide range of clinical manifestations and any organ system can be affected with varied disease severity. Generally, GPA can be divided into a limited (localised) or a general (systemic) form. However, it should be noted that clinically, both terms carry little significance; and that the terms are only widely used in clinical trials to classify patients for treatment options and in medical literatures. To date, there is no current consensus on the characteristics of these two groups, which clearly define them.

Limited GPA was first described by Carrington and Liebow in 1966 as the identical clinical onset and pathologic manifestations to the classic form i.e. the general or systemic form of the disease except in the absence of renal involvement (Carrington and Liebow, 1966). This term was used by the Wegener's Granulomatosis Etanercept Trial Research (WGET) group in their clinical trial for patients fulfilling the American College of Rheumatology (ACR) criteria for GPA, but lack features that pose immediate threat to either a critical individual organ or to the patient's life(WGET Research Group, 2002)(Stone, 2003).

The term "localised GPA" has not been generally accepted worldwide although it has also been used in many medical trails and literatures. In the European Vasculitis Study Group/The European League Against Rheumatism (EUVAS/EULAR) group, localised GPA was defined as individuals with vasculitic features confined only to the upper and/or lower respiratory systems, including the orbit (Hellmich et al., 2007)(Jayne and Rasmussen, 1997). In general, patients with limited or localised GPA are usually considered to have a

milder course of the disease and by and large respond well to less aggressive treatment regimens (Tarabishy et al., 2010)(Cassan et al., 1970).

In contrast, general (systemic) GPA displays a more severe form of the disease with a more widespread organ system involvement. Severe GPA in the WGET encompassed patients with significant involvement of vital organs that is potentially life threatening(WGET Research Group, 2002)(Stone, 2003).The EUVAS/EULAR divided patients with general GPA into early systemic, generalised, severe and refractory (Hellmich et al., 2007)(Jayne and Rasmussen, 1997).

The ocular and orbital structures involvement is common in patients with both limited and general forms of GPA, and may be the presenting feature in both. A very limited form of GPA with localised ophthalmic features only, has also been described (Ahmad et al., 2000). There appears to be no difference between the spectrum of ocular manifestations and incidence of potentially sight threatening complications seen in either the general or limited forms of GPA(STAVROU et al., 1993).

1.5 Pathogenesis of GPA

The exact pathogenesis of GPA is still unknown. Pathologically, GPA is described as a triad of granulomatous inflammation (inflammation with granuloma formation), vessel wall inflammation of small- to medium sized- vessel (vasculitis) and tissue necrosis. Many factors have been shown to potentially cause these pathological changes seen in GPA where some may have a more significant role than others. Indeed, the mechanism of GPA development may be a combination of several factors.

1.5.1 **Anti-neutrophil cytoplasm antibodies (ANCA)**

ANCA have been shown to be strongly associated with AAV including GPA. ANCA are a group of Immunoglobulin G (IgG) antibodies directed against the cytoplasm of neutrophils granulocytes and monocytes, and their presence in serum can be detected by indirect immunofluorescence. In GPA, antigens proteinase 3 (PR3) and myeloperoxidase (MPO) are most commonly identified with the disease. ANCA targeting these antigens are therefore termed as PR3-ANCA and MPO-ANCA.

In pattern of staining of ethanol-fixed neutrophils, ANCA is seen to exhibit two main patterns namely, c-ANCA (cytoplasmic antineutrophil cytoplasm antibodies) and p-ANCA (perinuclear antineutrophil cytoplasm antibodies).c-ANCA are mainly associated with sera containing PR3-ANCA and is closely associated with GPA. These neutrophil cells feature a diffusely granular cytoplasmic staining pattern. In contrast, p-ANCA, found mainly in sera containing MPO-ANCA, is observed to show a neutrophil perinuclear staining pattern and is strongly associated with microscopic polyangiitis (MPA). Both these antigens can be detected by enzyme-linked immunosorbent assay (*ELISA*). (Figure 1.1)

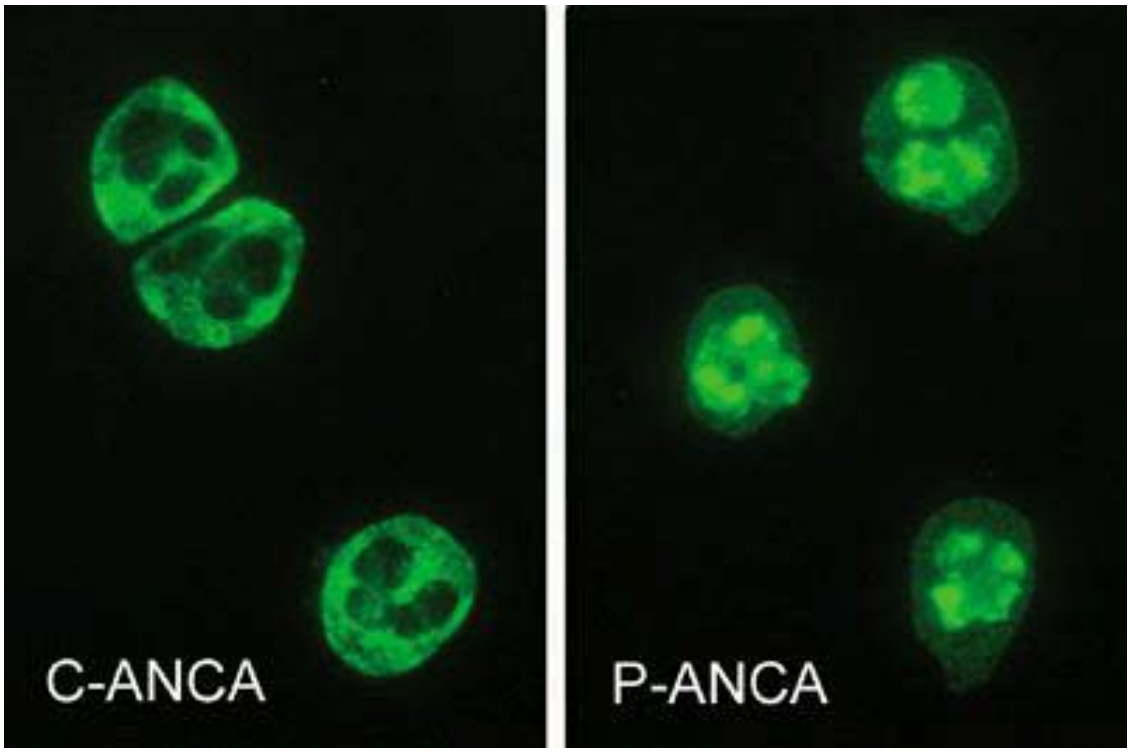

Figure 1.1: Cytoplasmic staining PR3 (c-ANCA) and perinuclear staining MPO (p-ANCA) seen via indirect immunofluorescence microscopy

In systemic GPA, c-ANCA are found in about 90% of patients, and in 80-95% of these patients the antibody is directed against PR3 (Tarabishy et al., 2010)(Lamprecht and Gross, 2007). c-ANCA is reported to have a 91% sensitivity and 99% specificity for active GPA (Taylor et al., 2007).

The exact mechanism in the generation of ANCA is still undetermined. One theory suggested that the ANCA production is an autoantibody response to newly exposed epitomes of target auto antigen at the site of initial tissue injuries (Radice and Sinico, 2005). Another theory proposed that ANCA generation is initiated in response to complimentary peptides that arise from the same gene molecule that encodes PR3 (Bautz et al., 2008),although another study failed to find the same association (Tadema 2011).

Despite the established association between GPA and ANCA, the aetiology and exact role of ANCA in the pathogenesis of GPA is still uncertain and studies in this area are on-going. In *in vitro* studies, ANCA is shown to activate primed neutrophils, promote their adherence to and transmigration through tumour necrosis factor (TNF)-stimulated endothelium. This antibody also promotes neutrophil degranulation which subsequently releases reactive oxygen species and lytic enzymes such as elastase, resulting in vascular endothelial cell and tissue damages. This phenomenon is thought to be accountable for the extensive damage to vessel walls which are seen in GPA (Seo and Stone, 2004). The alternative complement pathway which is an innate component of the immune system's natural defense against pathogens has also been shown to be involved in this process. The C3 complement, a protein of the immune system that plays a central role in the activation of the complement system, is found deposited in more than half of tissue biopsies in patients with AAV (Brons et al., 2001)(Vizjak et al., 2003).*In vitro*, activation of primed neutrophils by ANCA leads to the release of factor B, a component of the alternative complement pathway, as well as factor C3. This causes a further activation of the alternative complement pathway which releases the split product C5a protein. This protein, which acts as a strong chemotactic factor for neutrophils, is also able to prime neutrophils for the interaction with ANCA. Thus, a perpetual cycle for neutrophil recruitment and activation is formed, leading to continuing vascular inflammation and tissue damage (Kallenberg, 2011a).

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1.5.2 **Inflammatory cells associated with GPA**

The disease process i.e. inflammation in GPA is also believed to be an interplay of both the cellular (mainly T cells) and humoral (mainly B cells) functions of the immune system. T and B lymphocytes are both derived from the same hematopoietic stem cells and only

become morphologically distinct upon activation. These leucocytes (white blood cells) are the main effector cells in the human adaptive immune system. The adaptive immune system is the body's acquired immune defense system that develops after exposure to different antigens during its lifetime. In contrast, the innate immunity is the pre-existing immune defense system in the body which is encoded into the human germline. The adaptive immune system can be divided into the cell mediated or innate immune response, and the humoral or antibody immune response.

The cellular immune response is also known as the cell mediated immunity. T cells are mainly involved in this process, which, as the name reflects, largely involves the activation of specific cells such as neutrophils, lymphocytes, phagocytes and the release of various inflammatory cell mediators known as cytokines in response to an antigen upon pathogens invasion or tissue insult. B cells in contrast are involved in the humoral or antibody immune response where substances involved in the immune process are found in bodily fluids or "humours". The principle function of B cells is in the production of specific antibodies in response to specific antigens, hence the term "antibody mediated system". Apart from this, this arm of the immune system also produces larger molecules involved in the immune response such as complement factors or proteins.

Both T and B lymphocytes have been implicated in the development of GPA. The granulomatous inflammation seen in different tissues in GPA suggests a strong role of the T cell response where various cells are seen to be involved. T cell levels have been shown to be increased in patients with active GPA (Lúdvíksson et al., 1998)(Marinaki et al., 2006). Specific sub-types of T cells such as CD4 and CD8, and their sub-types such as CD8+CD57+ and NK-like CD4 cells, have been investigated in the pathogenesis of AAV, but their roles have not yet been clearly defined (Iking-Konert et al., 2009)(de Menthon et al., 2011). Recently, both the T-helper 17 (Th1 T cells7) cells and regulatory T (Treg) cells are also receiving attention as potential players in disease development in AAV, including

GPA (Berden et al., 2009)(Kallenberg). The role of B lymphocytes and memory plasma cells in immune regulation, antibody production and pathogenesis has now been well established in various auto-immune diseases(Yoshida et al., 2010)(Dörner et al., 2009). Plasma B cells also have been demonstrated to have a role in the production of autoantibodies, including ANCA (Jennette et al., 2006)(Fervenza, 2010). In addition, activated peripheral B cells have also been linked to disease activity and severity in GPA (Popa et al., 1999).Thus, treatment regimens targeting B cells like Rituximab have been advocated in the management of AAVs (Khan et al., 2010)(Onal et al., 2008a).

The granulomatous nature in the pathology of GPA implies a predominant role of macrophages in GPA. Active involvements of macrophages have indeed been reported in several studies (Mackiewicz et al., 2005) (Park et al., 2012)

1.5.2.1 T cells

T cells can be divided into several subsets where each generally has a distinct function. The subsets of T cells include the helper cells, cytotoxic cells, memory cells, regulatory cells and natural killer T cells.

1.5.2.1.1 T Helper T cells (Th)

T helper cells express the CD4 glycoprotein on their surface, therefore they are also known as CD4+ T cells. However, it is important to note that not all CD4 T cells are T helper cells as CD4 can also exhibit cytotoxic and suppressor cell function. CD4 T helper cells are activated upon the presentation of antigens by the antigen presenting cells (APC) via the major histocompatibility complex (MHC) class II molecules, and are differentiated

into two main subtypes, namely T- helper 1 (Th1 T cells) and T-helper 2 (Th2 T cells), distinguished by the cytokines they secrete, as well as other subtypes such as, T-helper 17 (Th17) and T-helper αβ. During an immune response, CD4 T-cells is directed to differentiate into a specific Th subtype via signaling from APCs and, by secreting specific cytokines, aid (hence “helper cells”) other immune cells function such as B cell maturation and activation of cytotoxic T cells (CD8) and macrophages. In granulomatous inflammation such as GPA, CD4 has been shown to have a key role in the inflammatory process as well as initiating granuloma formation and maintaining it via production of cytokines interferon-γ (IFN- γ), interleukin-12 (IL-12) and tumour necrosis factor (TNF) (Sneller, 2002)(Hänsch et al., 1996).

1.5.2.1.1.1 T Helper 1 T Cells (Th 1 T cells)

Th1 T cells secretes Interleukin (IL) 2, interferon-γ (IFN- γ) and tumour necrosis factor-α (TNF-α). It is triggered by IL-12, IL2 and its own cytokine INF-γ. The immune function of Th1 T cells is against intracellular bacteria, virus and protozoa by activating macrophages and cytotoxic T cells to kill microbes and infected cells. IFN- γ can also produce free radicals that directly kill intracellular bacteria and protozoa. Apart from this, Th1 T cells also stimulate B cells to produce specific IgG antibodies which cover the microbes and activate complements. Over-activity of Th1 T cells leads to over stimulation of immune cells such as macrophages and lymphocytes, causing chronic inflammation and persistent cytokine. This is known as Type 4 (cell mediated) hypersensitivity or delayed type hypersensitivity release. Examples of diseases in this group are Type 1 diabetes mellitus, multiple sclerosis and tuberculin reaction (Mantoux test) in the diagnosis of tuberculosis.

1.5.2.1.1.2 T Helper 2 T Cells (Th 2 T cells)

Th2 T cells secreteIL-4, 5, 10 and 13 and acts on extracellular pathogens such as multicellular helminths. Its differentiation is triggered by IL-4, IL-5 and IL-13. Th2 T cells can also stimulate B cells into producing most antibodies but in particular IgE production (Romagnani, 1991). Effector cells stimulated by Th2 T cells are eosinophils, mast cells and basophils. In helminths infection, IL-5 activates eosinophils to eradicate the pathogen. IL-4, together with IgE can result in the release of histamine, serotonin and leukotriene via mast cell stimulation that causes gastric fluid acidification and gastric peristalsis to expel the parasite. Over action of Th2 T cells response can lead to Type 1 IgE mediated allergy and hypersensitivity such as allergic rhinitis, asthma and atopic dermatitis.

1.5.2.1.1.3 T Helper 17 T Cells (Th 17 T cells)

Th17, a subset of T helper cells, are considered to be a distinct effector lineage from that of Th1 T cells and Th2 T cells lineage (Harrington et al., 2005). It is triggered by IL-6 and tumour growth factor beta (TGFβ). Th17 produces cytokine Interleukin 17 (IL-17), a pro-inflammatory cytokine as well as IL-21 and IL-22. It has been postulated that IL-23 may be involved in the expansion of Th17 populations and may, but not conclusively proven, to produce cytokine IL-17 (Bettelli et al., 2006). IL-17 has an important role in inducing and mediating pro-inflammatory responses by enhancing T cell priming, and stimulates the production of other pro-inflammatory molecules such as IL-1, IL-6, and tumour necrosis factor TNF.(Figure 1.2) Th17 has been reported to be involved in many auto-immune and chronic inflammatory diseases such as rheumatoid arthritis (Choy, 2012), psoriasis (Mudigonda et al., 2012)(Tokura, 2012), Crohn's disease and systemic lupus erythematosus (SLE) (Alunno et al., 2012). In the eye, Th17 is shown to play a role in ocular inflammation such as uveitis and scleritis (Amadi-Obi et al., 2007), Bechet's uveitis

(Sugita et al., 2012)(Shimizu et al., 2012) and uveitis associated with Vogt Koyangi Harada disease (Chi et al., 2007). In sarcoidosis, bronchus tissue granulomas and in circulating memory T cells have been reported to have an increased expression of IL-17A and are suggested to be involved in granulomas induction and maintenance (Ten Berge et al., 2012)

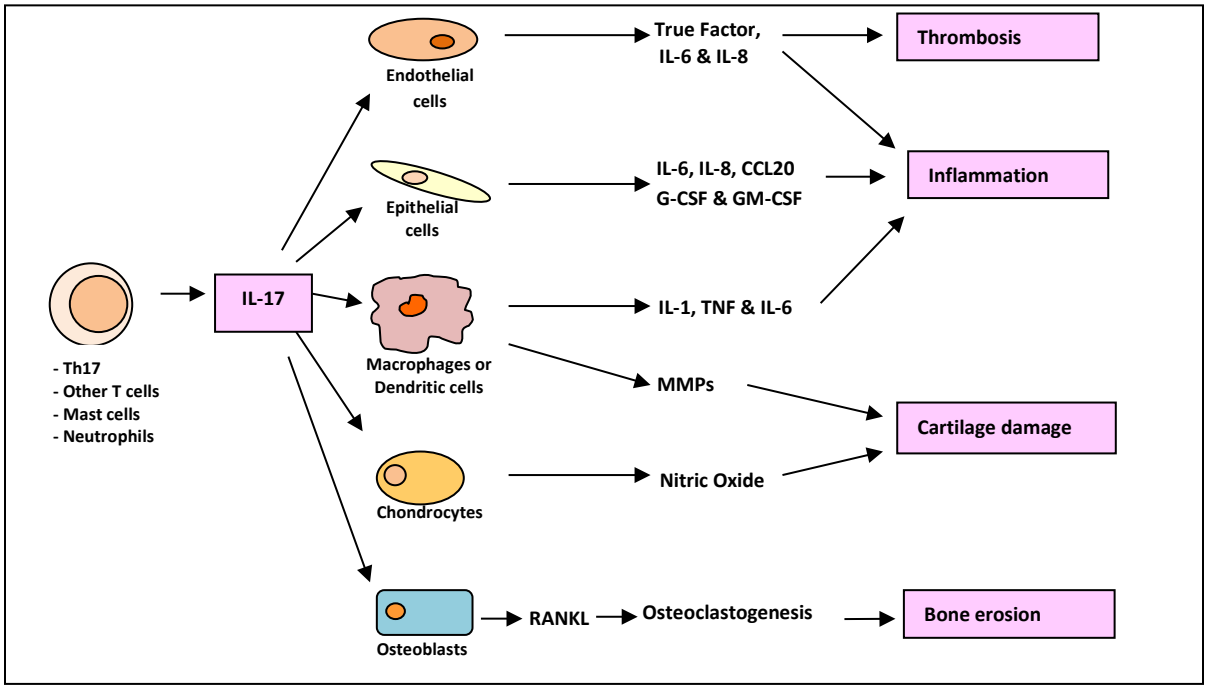


Figure 1.2: The role of IL-17 in inflammation. The effect of IL-17 on endothelial cells, epithelial cells and macrophages leads to production of further proinflammatory cytokines such as IL-1, IL-6, TNF and GM-CSF resulting in local tissue inflammation. In joints, macrophage, chondrocyte and osteoblast activation by IL-17 may results in cartilage damage and bone erosion such as seen in rheumatoid arthritis

1.5.2.1.1.4 T Helper αβ T cells (Th αβ T cells)

The differentiation of Thαβ helper cells is triggered by IFN α/β or IL-10. These cells function against viruses via their main effector, cytokine IL-10. ((Hu, 2013) The main effector cells activated by Thαβ helper cells are natural killer (NK) cells, CD8 T cells and IgG B cells. Activated NK cells apoptose virus-infected cells and causes viral as well as

host DNA fragmentation. Over-activation of THαβ can cause type 2 antibody-dependent cytotoxic hypersensitivity such as diseases like Myasthenia gravis or Graves' disease.

1.5.2.1.2 Cytotoxic T cells

Cytotoxic T cells are generally regarded as CD8 T cells, since these cells express the CD8 glycoprotein. Nevertheless, the CD8 protein co-receptor can also be found on other immune cells such as natural killer cells, cortical thymocytes and dendritic cells. In contrast to CD4 cells, these cells recognise antigens via the MHC class I molecules, found on all nucleated cell surfaces. These cytotoxic cells, as the name suggests, have the ability to destroy foreign cells including tumour cells and infected cells.

1.5.2.1.3 Memory T cells

Memory T cells are antigen specific T cells that persist in circulation even after the exposure to the antigen has subsided. Upon re-exposure to the specific antigen, these cells provides memory against the antigen, thus expands rapidly and exponentially. Cell surface CD45RO is generally considered to be expressed memory cells and can be either CD4 or CD8 T cells (Miyara et al., 2011)

1.5.2.1.4 Regulatory T cells (Treg cells)

Regulatory T cells have an important function in controlling the immune process. These cells are also known as suppressor cells, where the main role of these cells is to stop T cell mediated immunity at the end of an immune response, and suppress auto-reactive T cells. There are two main types of Treg cells; naturally occuring, and induced Treg cells. Naturally occurring Tregs are produced in the thymus and the induced Tregs generally

develop during an immune response. Naturally occurring Tregs are recognised from other T cells by the intracellular molecule FoxP3. Defects or mutation of the FoxP3 gene may cause Treg dysfunction that can lead to autoimmune diseases (Miyara et al., 2011).

1.5.2.1.5 Natural killer cells

Natural killer T cells are distinct from CD4 and CD8 as they do not recognise the MHC molecules, but recognise the glycolipid antigen presented by the CD1d molecule. It constitutes 0.1% of all T lymphocytes (Terabe and Berzofsky, 2007). These cells also have the ability to recognise tumour cells and herpes infected cells and destroying them (Novakova et al., 2012).

1.5.2.2 B cells

B cells are a component of the lymphocytic white blood cells which mature in the bone marrow, hence the term “B”-cells. It is the main effector cell in the humoral (or antibody) adaptive immune system. B cells can be distinguished from other lymphocyte cells such as T cells and natural killer cells by the cell surface B-cell receptor (BCR), which allows them to bind to specific antigens.

BCRs are highly specialised receptor proteins that allow only one specific antigen binding. Different types of B cells are continuously produced in the bone marrow where the BCRs on cell membrane have the ability to evolve and change over time. The B-lymphocyte antigen CD20 is an activated-glycosylated-phosphoprotein. This protein is expressed on the surface of all B cells; from immature pro-B cells to mature memory B cells. Its function is to facilitate optimum B-cell immune response, specifically against T-independent antigens via antibody production. The CD20 protein complex is generally used as a specific B cell marker and is expressed on all B cells, during all stages of development.

B cells and T cells are mainly distinct different by the way they recognise antigens. B cells recognise antigens in its naive form i.e. unbound and free in blood and lymph. T cells in contrast, recognise antigens in their processed form, where the antigen has been "processed" by antigen presenting cells APC's and then presented to the T cell via the MHC molecule.

1.5.2.2.1 B cell Function

The main response of B cells during an immune process is by the production of specific antibodies, which are produced in abundance for a specific antigen.

B cells exist as clones and all B cells are derived or "cloned" from a particular activated B cell. These cloned B cells have the ability to recognise the same antigen that activated the original B cell they originated from, and will have the same surface protein to bind with this specific antigen. The B cells ability to clone itself is the basis of the human immunogenic memory, one of the most important functions of the adaptive human immune defense system.

Upon encountering its specific antigen, a single B cell or a clone of cells with shared specificity, exponentially divides to produce numerous B cells. The majority of such B cells produced will differentiate into plasma cells for further antibodies production that will help in eradicating the pathogen. The remaining minority becomes memory cells that can recognize the exact same epitope of the antigen. In each cycle, the number of memory cells proliferates and with each cycle, the B cells' affinity to the particular antigen matures. The increased in number of cells and increased efficiency and specificity is known as the "secondary immune response". Fully differentiated and functioning B cells are called effector B cells, and B cells that are not yet activated are termed "naive lymphocytes".

1.5.2.2.2 The Different Types of B Cells

1.5.2.2.2.1 Plasma B cells (Plasma cells, plasmocytes or effector B cells)

Plasma cells are responsible for the production and secretion of large amounts of antibodies upon exposure to an antigen, sometimes referred to as antibody factories. These large B cells also play a role in the destruction of microbes by binding with them, thus making the pathogen become easier targets for phagocytes. Intracellularly, plasma cells contain large amounts of rough endoplasmic reticulum essential for the synthesis the antibody. Plasma cells are short-lived cells and undergo apoptosis once the inciting pathogen is abolished.

1.5.2.2.2.2 Memory B cells

Memory B cells are derived or cloned from activated B cells. The original activated B cells are highly specific to a particular antigen encountered during the primary immune response. Memory cells are long lived and have the ability to respond quickly following a further exposure to the same antigen (second immune response).

1.5.2.2.2.3 B-1 Cells

These B cells have receptors that show polyspecificity and expresses IgM antibodies more than IgG. This polyspecificity receptors means that these cells have low affinities for many different antigens. Polyspecific immunoglobulins often have a preference for other immunoglobulins, self-antigens, and common bacterial polysaccharides. B-1 cells are found predominantly in the peritoneal and pleural cavities, and some in the lymph nodes and spleen.

1.5.2.2.2.4 B-2 Cells

B-2 cells are conventional B lymphocytes in the circulation; produced postnatally (unlike fetal B-1 cells).

1.5.2.2.2.5 Marginal-zone B Cells

These are non-circulating B cells that are found in the marginal zone of the spleen. They can be recruited into the early adaptive immune response, and due to its location, it is ideal for defense against blood-bourne antigens.

1.5.2.2.2.6 Follicular B Cells

Follicular B cells exist in the primary and secondary follicles of lymphoid organs such as the spleen and lymph nodes. These cells can freely circulate in the body and make up 95% of B cells in the lymph nodes. Together with T cells, they assist in promoting the primary immune response.

1.5.2.2.2.7 Regulatory B cells (Breg cells)

Bregs are involved in immune regulation. This is done via various mechanisms including secretion of IL-10 and TGF β (Yang et al., 2013). Sub-sets of Bregs can be found within both the B-1 and B-2 cell population.

1.5.2.2.3 B Cells Activation

B cells ability to produce antibodies can both be dependent or independent of the T cell activity.

1.5.2.2.3.1 T Cell Dependent B Cell Activation

The production of antibodies by activated B cells can be under the influence of T cell activity. Upon pathogen detection and ingestion by macrophages or dendritic cells, the proteins of the ingested pathogen are attached to MHC class II peptide where this complex sits on the cell surface. This complex in turn activates T cells resulting in the proliferation of T effector and memory cells. In addition, T helper cells activate specific B cells to produce antibodies. These antibodies subsequently surround and inhibit the pathogens until it is eradicated by phagocytes or the complement system.

1.5.2.2.3.2 T Cell Independent B Cell Activation

Type 1 T cell-independent activation relies upon toll-like receptors e.g. TLR9 for DNA. The activation occurs when B cells that are already bound to antigens, are given a secondary activation by these TLR. These activated B cells produced are specific to the TLR-binding antigen and are only able to produce IgM antibodies.

Type 2 T cell-independent activation occurs when B cells are exposed to pathogens with an organized and repetitive form, like bacteria. Activation is achieved by the cross-linking of the antigen receptors in a multivalent fashion. This leads to IgM synthesis in the absence of T cell stimulation.

1.5.2.2.4 B Cell Maintenance

BAFF is a cytokine that belongs to the tumour necrosis factor (TNF) ligand family encoded by the *TNFSF13B* gene that has been shown to have a critical role in B cell survival and maintenance(Schneider et al., 1999). It is also known as B Lymphocyte Stimulator (BLyS). This cytokine is expressed in B cell lineage cells but is also found on monocytes, dendritic cells and bone marrow stromal cells. BAFF is a potent B cell activator with a key role in the proliferation and differentiation of B cells(Mackay et al., 2003)(Kalled et al., 2003). In the human immune response, it acts potentially as a regulator of B and T cells functions.

The steady state of BAFF relies on B cells and also on BAFF-binding receptors expression (Kreuzaler et al., 2012). BAFF naturally binds with three TNF-receptors, expressed on both immature and mature B lymphocytes. These TNF receptors are BAFF-R (BR3), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), and BCMA (B-cell maturation antigen). BAFF has differing affinities to these receptors with BAFF-R having the higher affinity to BAFF compared to TACI and BCMA. BCMA, which is also expressed on plasma cells, has the worst affinity to BAFF, binding better with a protein similar to BAFF called a proliferation-inducing ligand (APRIL). TACI has an intermediate binding affinity and interacts with either BAFF or APRIL (Bossen and Schneider, 2006). TACI can also be found expressed on sub-sets of T cells.

BAFF-R in particular, has a role in the positive regulation of B cell development, which is critical for B cells' survival, germinal center maintenance and antibody production (Thibault-Espitia et al., 2012)(Kalled, 2006). The BAFF/ BAFF-R interaction activates the NF-κB signaling pathways which stimulates B lymphocytes to undergo proliferation and to counter apoptosis. Thus, this interaction is crucial for the formation and maintenance of B cells and is important for a B-cell survival(Bossen and Schneider, 2006)(Kreuzaler et al., 2012).

APRIL is another cytokine that belongs to the tumour necrosis factor (TNF) ligand family with a high affinity to TACI and BCMA receptors. APRIL, like BAFF, is also found to be important for B cell development particularly in the long-term survival of plasma B cells in the bone marrow(Bossen and Schneider, 2006).

1.5.2.2.5 BAFF and APRIL T Cells Maintenance

BAFF and APRIL are generally recognised for their function in B cells survival and maturation, yet BAFF and APRIL are also expressed by T lymphocytes. BAFF and APRIL appear to also promote T cell activation and survival(Leandro and Cambridge, 2013)(Zhao et al., 2012)(Ramos-Casals, 2013)(Saussine et al., 2012). In animal studies, BAFF expression has been shown to enhance Th1 T cells driven hypersensitivity but inhibits Th2 T cell mediated pathways. This suggests that interaction of BAFF on T cells may also play a role in immunomodulation and inflammation (Mackay et al., 2005).

1.5.2.2.6 Clinical Significance of BAFF

The immunostimulant activity of BAFF appears to be necessary for maintaining normal immunity. Inadequate levels of BAFF which leads to failure in B cells activation, reduces immunoglobulin production that can result in immunodeficiency. In infections, BAFF is said to also have a role in priming or enhancing B and T cell activity to assist in microbial eradication (Mackay et al., 2003). On the other hand, excessive levels of BAFF will cause abnormally high antibody production, which is the basis of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Roschke et al., 2002).

1.5.2.3 Macrophages

Macrophages are white blood cells found in tissues and are part of the mononuclear phagocyte system. In humans, they are about 21µm in diameter. They are derived from differentiation of circulating monocytes and have a role in both the innate (non-specific defense system) and acquired (specific defense system) immune system. These phagocytic cells have the capacity to regenerate and are found in all living tissues.

Macrophages have the unique ability to either kill or repair. This is done by metabolising just a single amino acid molecule, arginine, to either form nitrix oxide (killer molecule) or ornithine (repair molecule) (Mills, 2012). Killer macrophages are generally known as M1 macrophages. They are activated by the lipopolysaccharides (LP), which are molecules found on the cell surfaces of gram negative bacteria and IFN-gamma, and secrete mainly IL-12 cytokine. They are active in inflammation and produce a small amount of IL-10, an anti-inflammatory cytokine. In contrast, M2 macrophages are involved in tissue repair. Activation of these molecules is by IL-10 and IL-4, and they in turn produce more IL-10, TGF- beta and small amounts of IL-12. Despite somewhat appearing to have a ”protective” mechanism in the body, M2 macrophages however have been associated with promoting tumour growth (Galdiero et al., 2013). M1 and M2 macrophages are also known as the classical (M1) and alternative (M2) activated macrophages.

However, in general, macrophages are recognised to have three main primary functions which are; 1) as scavengers to eradicate pathogens and clear debris, 2) as an antigen presenting cell (AP) for other immune cells such as T cells and 3) as one of the effector cells during the inflammatory process. Pathogen ingestion by macrophages is termed “phagocytosis”. Upon encountering a pathogen, a macrophage will initially ingest it. Once within the macrophage, the ingested pathogen becomes trapped in a phagosome. This phagosome, along with the trapped pathogen thereafter fuses with lysosome, which is a

spherical vesicle in the cytoplasm of the macrophage containing acid hydrolase. Acid hydrolase has the ability to breakdown all kinds of biomolecules, including proteins, lipids, carbohydrates, nucleic acids and cellular debris. The trapped pathogen then becomes digested by the acid hydrolase and is eradicated. A single macrophage is able to digest more than 100 bacteria before finally dying from their own digestive compounds. However, not all pathogens are easily removed by this process. Mycobacterium tuberculosis is one bacterium known to be resistant to macrophage attacks.

Upon pathogen invasion, macrophages also stimulate other immune cells such as lymphocytes and eosinophils, by releasing various cytokines in response to the pathogen attack. In an event where a pathogen is unsuccessfully eradicated and antigens persist, for example in the case of mycobacterium tuberculosis, macrophages will accumulate it and together with other immune cells, surround the foreign material in an attempt to wall it off. The formation of densely packed immune cells form a ball-like structure called granuloma, which are typically seen in tuberculosis. As previously described, inflammation with granuloma-like features or predominance of mononuclear phagocytic cells, is generally termed “granulomatous inflammation”.

In the acquired immune system, macrophages also act as an antigen presenting cell (APC) to lymphocytes and other immune cells. They can also be activated into effector cells, releasing further inflammatory mediators and eradicating pathogens via phagocytosis. At some inflammation sites, activated macrophages can morphologically transform to resemble epithelial cells, hence termed “epithelioid cells”, seen particularly in granulomatous inflammation. These epithelioid cells may fuse to form multinucleated giant cells. Epithelioid cells as well as multinucleated giant cells are some of the histological features associated with GPA.

There are several cell phenotypes and cytokines identified and associated with the macrophage cell lineage (Mosser and Edwards, 2008).

1.5.2.3.1 CD68 Macrophages

CD68 is a glycoprotein which binds to low density lipoprotein, and is particularly useful as a marker for the various cells of the macrophage lineage, including monocytes, histiocytes, giant cells, Kupffer cells, and osteoclasts. It is generally the most common cell marker used to identify M1 macrophage presence in tissues.

1.5.2.3.2 CD163 Macrophages

CD163 has been shown to specifically mark cells of the monocytes/macrophage lineage (Lau et al., 2004). CD163 is a scavenger receptor for the haemoglobin-haptoglobin complex and is seen upregulated in several inflammatory diseases such as liver cirrhosis, type 2 diabetes and rheumatoid arthritis (Andersen et al., 2014)(Jude et al., 2013)(Fjeldborg et al., 2013).

1.5.2.3.3 CD204 Macrophages

The CD204 (macrophage scavenger receptor) is used as a marker of the alternative activation of macrophages (M2), and influences B cell auto-immunity by regulating soluble auto-antigen concentration (Haasken et al., 2013). CD204 together with CD163, have been associated in the development of chronic obstructive pulmonary diseases (Kaku et al., 2014). However, the roles of these cells have not been established in GPA.

1.5.2.3.4 Allograft inflammatory factor-1 (AIF-1) cytokine

Cytokines produced by macrophages have also been linked to the development of auto-immune diseases. Allograft inflammatory factor-1 (AIF-1) cytokine, expressed in macrophages, as well as neutrophils and also in a subset of CD68 macrophages (Utans et al., 1995), AIF-1 is said to have a critical role in the pathogenesis of auto-immune diseases such as rheumatoid arthritis, where it has been shown to have a role in vascular inflammation (Kimura et al., 2007). Its presence has not been investigated in GPA tissues.

1.5.2.3.5 IL-23 cytokine

Cytokine IL-23, a heterodimeric cytokine, is made up of two subunits: p40, also a component of the IL-12 cytokine, and p19, considered to be the IL-23 alpha sub-unit. IL-23 is produced by macrophages and dendritic cells, and plays an important role in bridging innate and adaptive responses. In chronic inflammations, IL-23 influences the expansion of CD4 T cells to produce IL-17 via Th17, a pro inflammatory cytokine previously described in Chapter 3. IL-23 also activates dendritic cells and macrophages to produce further proinflammatory cytokines such as IL-1, IL-6, and TNF-α. (Figure 1.3)In contrast to AIF-1, IL-23 has been reported to be elevated in patients with AAV, including GPA (Langrish et al., 2005). The presence of IL-23 in peripheral inflamed tissues has also not yet been demonstrated.

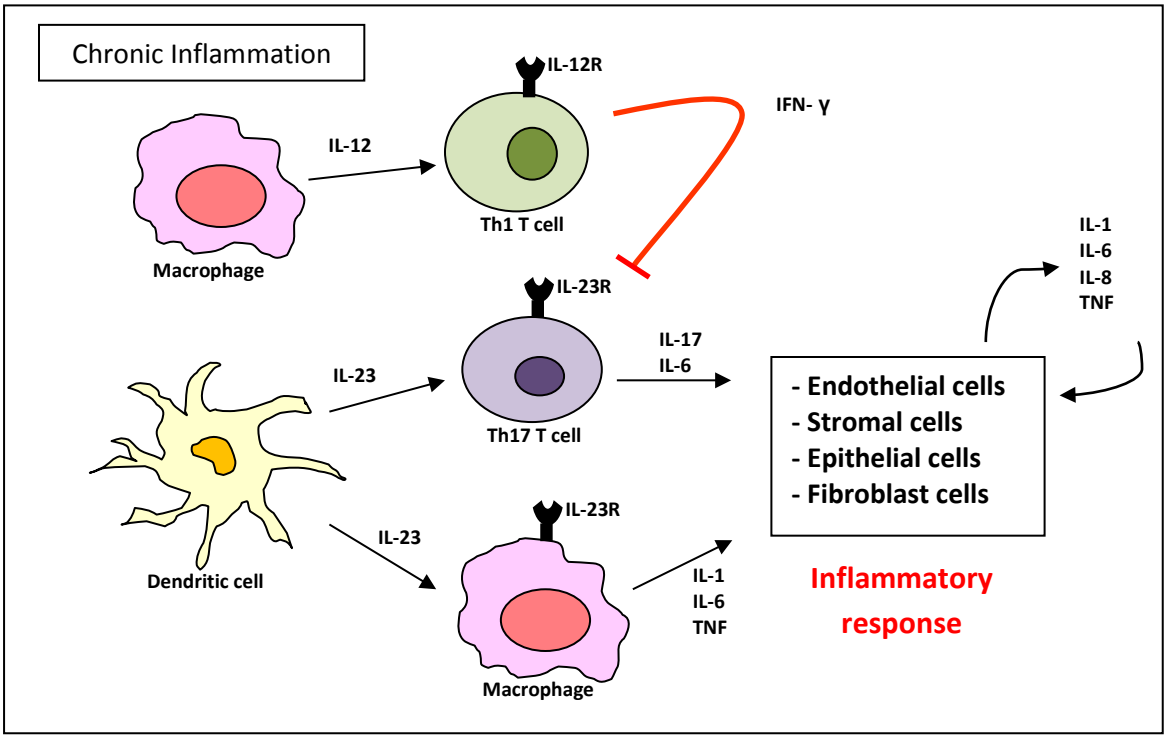


Figure 1.3: The IL-23/IL-17 axis in inflammation. IL-23 produced from macrophages and dendritic cells promotes the development of Th17 cells which produces IL-17 cytokines. The effect of IL-17 on endothelial, stroma, epithelial and fibroblast cells in turns enhances T cell priming and produces proinflammatory cytokines such as IL-1, IL-6 and TNF- α leading to an inflammatory response.

1.5.3 **Infection**

Staphylococcus aureus (*S. aureus*) has been implicated in the pathogenesis of AAV, particularly GPA with respiratory involvement, where up to 60-72% of patients were found to have nasal carriage of *S. aureus*(Zycinska et al., 2008)(Laudien et al., 2010). It is postulated that low-grade chronic infection in the upper airway causes release of pro-inflammatory cytokines that are able to prime neutrophils locally, which are then further activated by ANCA(Kallenberg, 2008). One of the mechanisms that have been suggested that links infection to GPA development is molecular mimicry. This is because GPA patients who are PR3-ANCA positive possess antibodies reactive with a protein produced from PR3-antisense RNA, and these proteins have been shown to have an amino acid

sequence that has homology with proteins from many microbes and viruses(Preston et al., 2005). In addition, the anti-human lysosomal membrane associated protein (LAMP) 2 antibodies, have also been shown to target adhesion elements, FimH (fimbriae) on gram negative bacteria. Interestingly, anti-LAMP2 antibodies have also been found to be present in 100% of AAV patients with active renal involvement and in 93% of a cohort of 84 patients with GPA, MPA or renal limited vasculitis. This again suggests a connection between infection and vasculitis through molecular mimicry (Kain et al., 2010). Nevertheless, there has been no general consensus on this finding and hence further confirmatory studies are required.

Recently, toll like receptors (TLRs) have also been implicated in the pathogenesis of autoimmune diseases such as SLE as well as GPA (Capolunghi et al., 2010). TLRs are important receptors on cells of the immune system which function by sensing invading pathogens like bacteria and viruses. They orchestrate the immune response during a pathogen invasion resulting in the release of pro-inflammatory cytokines, lymphocyte production and antibody production (Tadema et al., 2011). Expressions of TLR2 and TLR9 in particular, have been shown to be linked to GPA, as membrane PR3 expression is found to be heightened by TLR2 and TLR9 stimulation. In addition, TLR2 and TLR9 ligands also act as neutrophil priming agents similar to TNF-alpha, triggering ANCA mediated neutrophil activation. This is thought to further explain the role of infections in triggering disease activity in GPA (Holle et al., 2013a).

1.5.4 **Genetic factors**

Genetic associations with GPA have also been studied but results are generally inconclusive. The *HLA-DPB1-15* allele is reported to have a strong link with the

development of PR3 associated ANCA vasculitis (Lamprecht et al., 2009). Defective allele PI*Z on chromosome 14q32.1 has also been associated with disease development in ANCA diseases and rheumatoid arthritis (Borgmann et al., 2001).

1.5.5 **Drugs**

Some drugs have also been seen to be associated with the development of AAV. Treatment used in the treatment of thyroid disease and Graves' disease such as propylthiouracil and benzylthiouracil has indeed been shown to be inducers of this disease (Panamonta et al., 2008). However reports were mainly showing associations of these drugs with MPO-ANCA and not PR3-ANCA. Other drugs that have also been implicated in the induction of ANCA positive vasculitis include sulfasalazine, a drug used in the treatment of inflammatory bowel disease. In contrast to propylthiouracil and benzylthiouracil, sulfasalazine has been shown to actually induce and exacerbate GPA (Denissen et al., 2008). Hydralazine, commonly used in the treatment of hypertension and systemic lupus erythromatosus (SLE), has also been shown to cause ANCA positive renal vasculitis, resulting in acute nephritis (Keasberry et al., 2013).

1.5.6 **Environmental exposures**

Exposure to silica has also been shown to have a link in the development of AAV. The exact mechanism in which silica causes AAV is still unclear but it is suggested that silica particles in the trachea may accelerate the apoptosis of neutrophils by producing free radicals. The silica particles also activate alveolar macrophages that then produces inflammatory chemokine and cytokines which may result in immune modulating defect

(Chen and Kallenberg, 2010)(Gómez-Puerta et al., 2013). Vitamin D deficiency is another environmental factor that is proposed to cause GPA. Human bodies manufacture vitamin D with ultraviolet B rays, thus, it is postulated that, countries in the northern and southern hemisphere where GPA is prevalent, Vitamin D deficiencies are high due to the low ultraviolet radiation gradient there. Indeed it has been shown that vitamin D may reduce cytokines interleukin 6 (IL-6) and tumour necrosis factor –alpha (TNF-alpha) in an inflammatory environment (van Hamburg et al., 2012).

1.6 Clinical Presentation in GPA

1.6.1 General GPA

GPA can affect any organ system though the disease tends to affect the upper and lower respiratory tract and kidneys more, compared to other structures. However, the clinical presentation varies, and may be non-specific such as symptoms of fever, malaise, anorexia, weight loss and arthralgia (Gottschlich et al., 2006).

1.6.2 Upper and Lower Respiratory Manifestations of GPA

GPA has a predilection to affect the upper and lower respiratory tract systems where involvement of this system can occur in up to 85% of patients (Manganelli et al., 2006)(Hoffman et al., 1992). Despite this predilection, symptoms involving the upper respiratory are often non-specific. The most frequent initial presentation is chronic sinusitis that is unresponsive to treatment seen occurring in approximately 70% of patients with GPA. In these patients, recurrent inflammation of the nose could lead to damage to the

nasal mucosal lining resulting in dryness, crusting and epistaxis. Subsequently, patients then become susceptible to infection. As a result of repeated insults from infection and inflammation, extensive tissue damage such as nasal septal perforation, can occur. Cartilage destruction of the nasal bridge, which gives rise to the 'saddle nose deformity', is closely associated with GPA, particularly the retro-orbital mass in GPA (Gomes et al., 2010). (Figure 1.2)

Figure 1.4: Saddle nose deformity (arrow) from nasal septum destruction

In the ear, chronic otitis media is a common manifestation of GPA. Perforation or thickening of the tympanic membrane, ossicle fusion or damage to the neurosensory pathway in the ear resulting in hearing loss, may occur from recurrent ear infections and vasculitic inflammation in the middle ear. Oral involvement in GPA include gingivitis and ulcer, are seen in 10% of patients. In particular, strawberry gingivitis or hyperplastic granular gingivitis is characteristic presentation of GPA although this presentation is rare. Subglottic stenosis is a life threatening complication of GPA. GPA patients who present

dyspnoea in the absence of active pulmonary disease should be investigated for this condition and managed urgently.

Lower respiratory or pulmonary involvement can occur in about 45% of GPA patients with 85% eventually developing lung disease. Respiratory symptoms include dyspnoea, cough, chest pain and haemoptysis. Radiographically, the most common findings on chest X-Rays are solitary or multiple nodules and masses which mainly represent pulmonary granulomas with or without cavitations. Nevertheless, despite these findings, patients may still be asymptomatic(Manganelli et al., 2006). Other chest X-ray findings include fibrosis, lung consolidation and signs of secondary chest infection. Lung collapse, severe pneumonia; secondary either to the disease or immunosuppressive treatment, alveolar haemorrhage and obstruction, are severe pulmonary complications associated with GPA and can lead to death.

1.6.3 **Renal Manifestations of GPA**

Together with secondary infections, renal disease is the main cause of mortality in GPA(Singer et al., 1990)(Takala et al., 2008). Renal involvement occurs in 75-80% of patients with GPA, yet only 20% of patients, in a non-renal clinic, will have features of active glomerulonephritis (Takala et al., 2008). Clinical signs of renal involvement in GPA include proteinuria, microscopic haematuria and, in advanced cases, oliguria (Lamprecht and Gross, 2007). Histopathology from renal biopsies of patients with GPA commonly reveals focal and segmental pauci-immune crescentic glomerulonephritis with necrotic changes in the glomerular capillary loops seen as one of the earliest histologic abnormalities. Once the renal system is involved, the disease may progress rapidly although the patient may remain asymptomatic. Prognosis largely relates to serum

creatinine at presentation (Gottschlich et al., 2006). Rapidly progressive glomerulonephritis (RPGN) is the most severe renal manifestation of GPA. This condition can lead to the need for dialysis or transplantation within weeks if untreated. Another rare but serious complication related to ANCA-associated vasculitis, including GPA, is the pulmonary renal syndrome. This syndrome is a combination of diffuse alveolar haemorrhage and RPGN(Manganelli et al., 2006).

1.6.4 Musculoskeletal Manifestations of GPA

Musculoskeletal symptoms occur in approximately two-thirds of GPA patients, with migratory polyarthralgias and myalgias being the most frequent musculoskeletal complaint. Although less frequent, symmetrical polyarticular involvement can also resemble rheumatoid arthritis (RA). Asymmetric pattern and monoarticular pain are not common in GPA and unlike RA; arthritis in GPA does not cause deformities and is typically non-destructive.

1.6.5 Cardiovascular Manifestations of GPA

Myocardial infarction associated GPA often results from vasculitis of the coronary vessels (Cocco and Gasparyan, 2010). It has been reported in one study that GPA patients appear to have an increased morbidity from ischaemic heart disease compared to the rest of the population (Faurschou et al., 2009). In addition, GPA patients with cardiovascular manifestations seem to have an increased risk of first GPA relapse after initial remission (Pierrot-Deseilligny Despujol et al., 2010).

1.6.6 **Gastrointestinal Manifestations of GPA**

Gastrointestinal manifestations of GPA are now increasingly recognised as part of this disease. Clinically, patients may present with an acute abdominal pain with signs of peritonitis (Yildirim et al., 2010). Lesions are normally seen when performing colonoscopy or enteroscopy, manifesting as multiple intestinal ulcerations which can involve both the large and small bowels (Beppu et al., 2011)(Thanarajasingam et al., 2011). These ulcers may subsequently lead to multiple intestinal perforations (Beppu et al., 2011)(Yildirim et al., 2010). Other reported gastrointestinal lesions associated with GPA include vasculitis and multiple mesenteric lymphadenopathy (Jeong et al., 2010).

1.6.7 **Ocular GPA**

Ocular involvement occurs in 50-60% of patients with GPA (Joshi et al., 2009) and any ocular structure may be affected; from the eyelid and orbit to the optic nerve. Ocular GPA can either manifest *de novo*, as disease spread from contiguous structures like the sinuses, or as part of a more widespread systemic form of the disease. Ophthalmic presentations are also variable and can present as an orbital mass (orbital granuloma), ocular vasculitis, adnexal inflammation and nasolacrimal duct changes.

Ocular inflammation can occur with or without systemic manifestations of GPA (Harper et al., 2001). Visual loss is seen in up to 8% of patients where severe ocular morbidity can occur in both the limited and systemic forms of the disease. In 8-16% of patients, ocular manifestations may be the initial presentation of GPA. However, as the signs and symptoms are usually non-specific and tend to overlap with those of other orbital inflammatory disorders, diagnosis is often difficult (Pakrou et al., 2006)(Fortney and

Chodosh, 2002). It has been shown that patients with ocular GPA could progress to the systemic form in one study (Choopong et al., 2005).However, others have failed to observe the same pattern (Fechner et al., 2002)(Holle et al., 2010a).

1.6.8 **Orbital Manifestation of GPA**

The orbit is a common ocular structure involved in GPA. In the majority of cases, orbital involvement occurs as part of disease spread from adjacent structures such as the nose and paranasal sinuses already affected by the disease. Nevertheless, orbital GPA may also be the initial manifestation of the GPA, exhibiting either with orbital granuloma formation or orbital vasculitis. Common presentations of orbital GPA include proptosis from an orbital mass, periorbital swelling, eye redness and pain, reduced vision, diplopia and epiphora(Tarabishy et al., 2010)(Pakrou et al., 2006). (Figure 1.3)

Figure 1.5: Left eye proptosis (arrow) in orbital GPA

Apart from being the most common ocular presentation of orbital GPA (Tarabishy et al., 2010) proptosis is also an important sign. Together with respiratory diseases or glomerulonephritis, the combination of these symptoms is highly suggestive of the diagnosis of GPA(Hoffman et al., 1992). Patients with proptosis and lid destruction are susceptible to develop exposure keratopathy. This can further lead to the development of corneal ulceration, ocular perforation and blindness (Pakrou et al., 2006). Inflammation of the extraocular muscles or vasculitis of the vasa nervorum results in orbital myositis in GPA, where patients mainly present symptoms of diplopia and pain on ocular movement and show signs of restriction of ocular motility (Salam et al., 2008).

In orbital GPA, the optic nerve can be affected when there is infiltration of the disease in the orbital apex. In such cases, symptoms present in patients may include painless optic neuropathy, optic nerve swelling and ensuing atrophy.(Figure 1.4) As the disease further infiltrates, patients may develop painful ophthalmoplegia and blindness (Shunmugam et al., 2011).Vasculitis involving the cranial nerves supplying the extraocular muscles may also cause ophthalmoplegia(Pakrou et al., 2006).The orbital contracture syndrome, described as proptosis followed by the development of enophthalmos with radiographic evidence of fibrotic changes in the orbit, can also occur as a complication of orbital GPA. This complication is thought to be due to the proliferation of fibrous tissue replacing areas of acute inflammation and necrosis where ensuing contracture leads to restriction of motility and enophthalmos. The optic nerve may also be involved in this, resulting in further visual loss. As with other fibrosing manifestations of GPA, socket contracture responds poorly to immunosuppressive therapy (Talar-Williams et al., 2005).

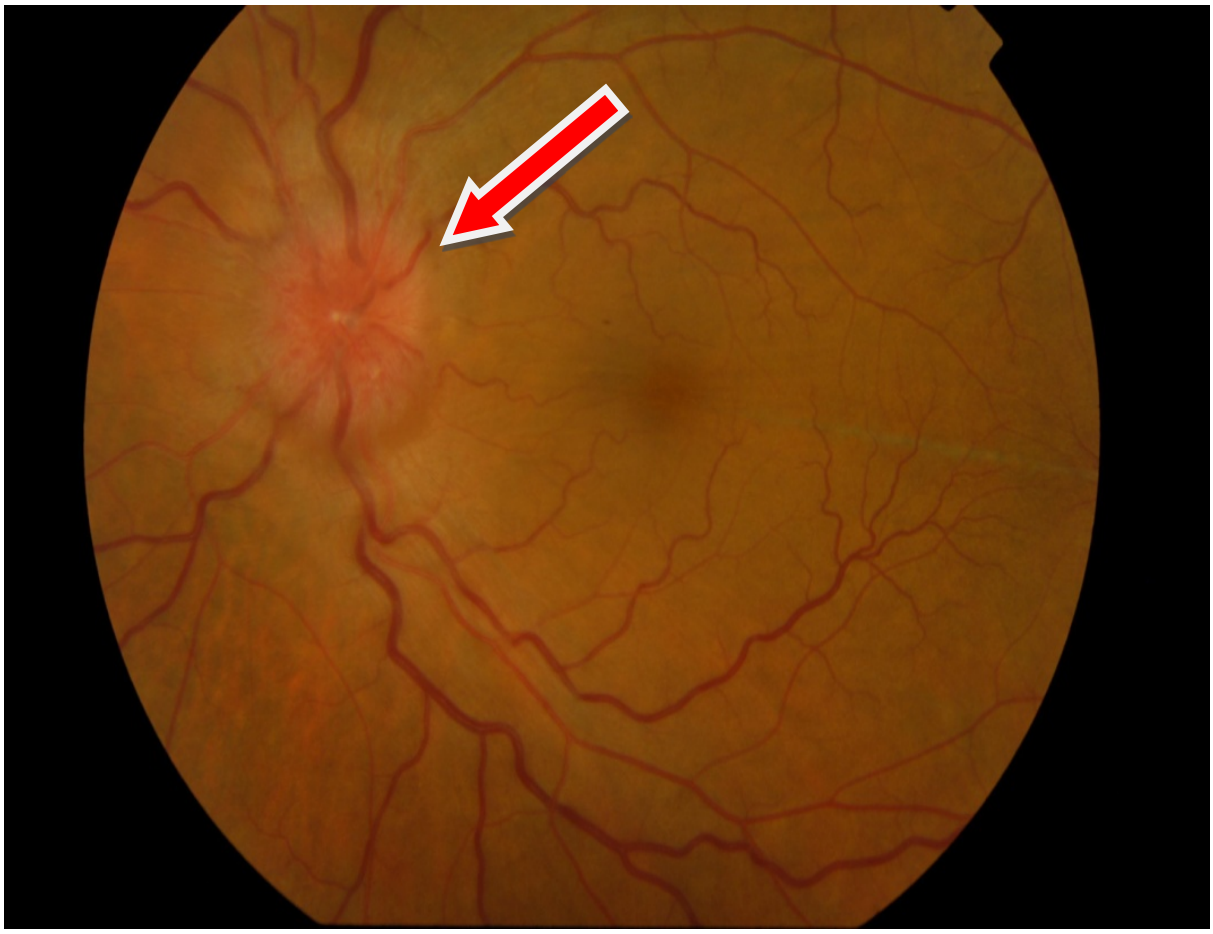


Figure 1.6: Left optic disc (arrow) swelling in orbital GPA

1.6.9 Sclera and Episcleral Manifestation of GPA

Scleritis involves inflammation of the entire thickness of the sclera and can manifest in two forms; necrotising scleritis, which is associated with necrotic features, or non-necrotising scleritis, which has no necrotic features. (Figure 1.5)Necrotising scleritis is one of the common ophthalmic presentations of GPA, occurring in 50% of patients (García et al., 2006)(Biswas et al., 2003) and may be the initial clinical presentation of GPA (Hijikata et al., 2009). (Figure 1.6)Typically patients present severe deep boring pain that may radiate

to the jaw and temple with red, tender eyes. The pain associated with scleritis typically becomes worse at night, often waking the patient from sleep. On ocular examination, areas of capillary non-perfusion due to severe vasculitis causing capillary closure in the deep episcleral vascular bed are seen and are characteristic of this disease. This can cause infarction and necrosis of the involved sclera, and exposure of the underlying uvea (Galor and Thorne, 2007). Despite scleral thinning, globe perforation is rare. However, adjacent ocular structure such as the cornea, trabecular meshwork and ciliary body, may also be involved during active inflammation and may result in complications such as keratitis, corneal ulceration, uveitis, ocular hypertension or glaucoma(Jabs et al., 2000).

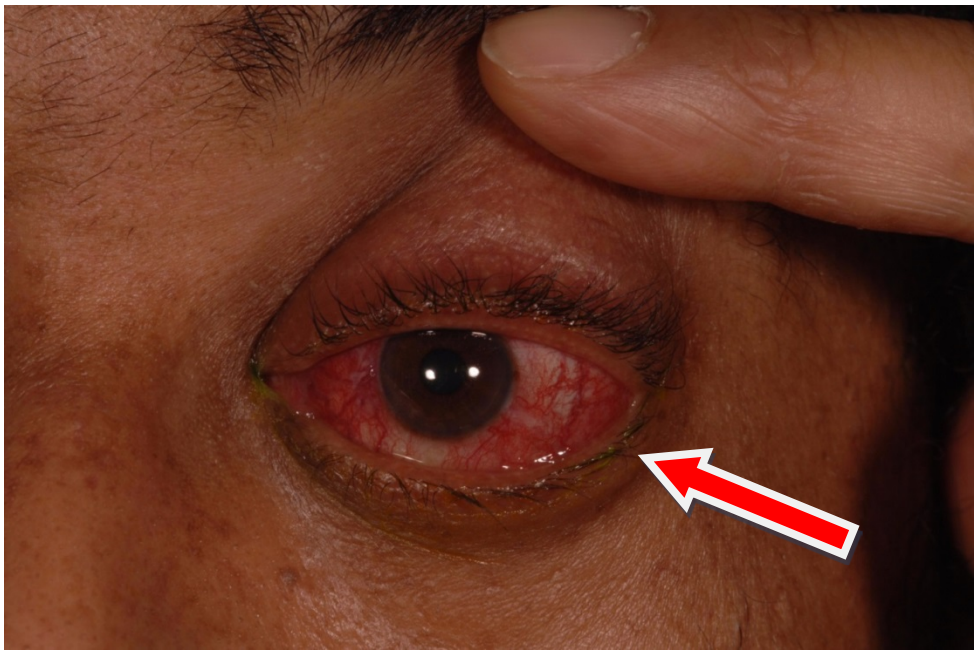


Figure 1.7:Left eye scleritis in GPA

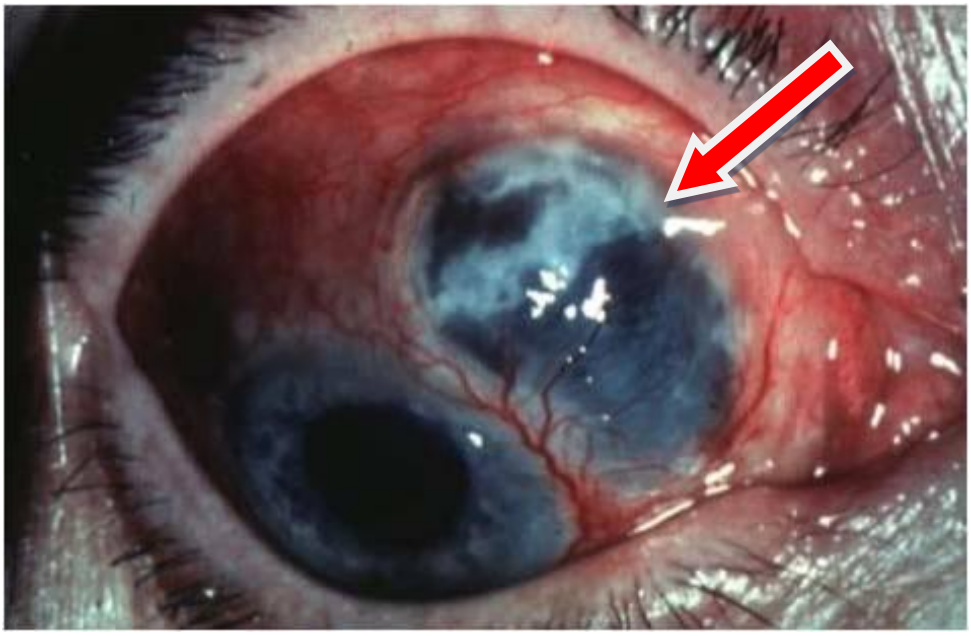


Figure 1.8: Necrotising scleritis in GPA. Arrow showing the area of sclera thinning secondary to tissue necrosis showing the underlying uvea tissue.

Episcleritis, characterised as inflammation of the loose episcleral tissues between the conjunctiva and sclera (Pakrou et al., 2006) has also been described in patients with GPA (Tojima et al., 1994) although not usually associated with systemic diseases. Its presentation is usually less severe compared to scleritis. Patients may complain of red eye with mild discomfort and epiphora. Ocular examination typically reveals an eye which is either diffusely or locally injected. Episcleritis generally runs a milder course compared to scleritis and is frequently self-limiting with few or no ocular complications. In GPA however, a short course of topical glucocorticoids for patients with episcleritis is often necessary(Tarabishy et al., 2010).

1.6.10 **Cornea Manifestation of GPA**

Peripheral ulcerative keratitis (PUK) is another frequent ocular manifestation of GPA(García et al., 2006) and cases of bilateral eye involvement has been reported in the past (Chan et al., 2007). (Figure 1.7)

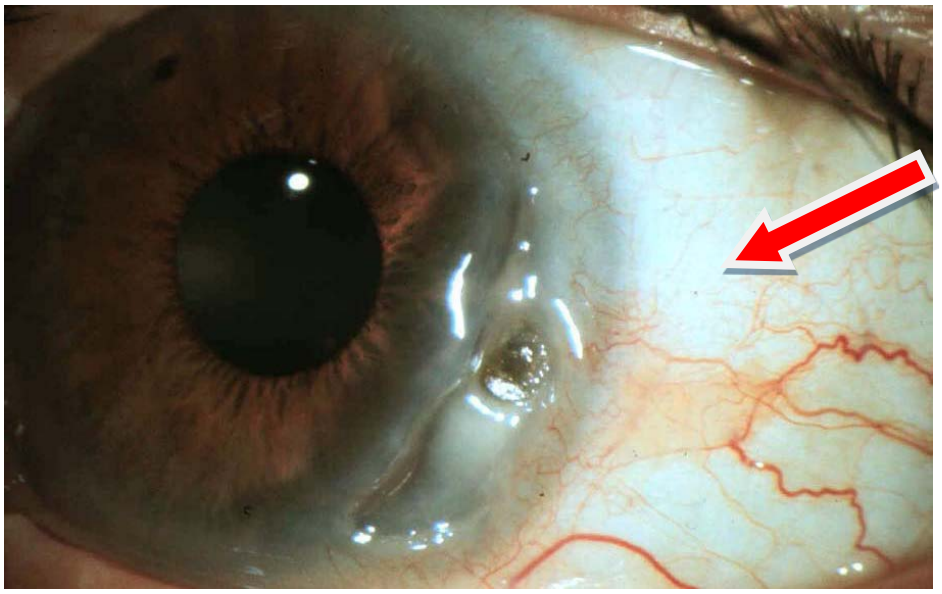


Figure 1.9: Peripheral ulcerative keratitis (arrow) in GPA

Patients presenting with PUK often have concurrent scleritis. In this situation, ocular pain may be severe and other symptoms may include photophobia, tearing and poor vision. On ocular examination, corneal stromal infiltration and clouding, with invading vessels from the limbus are usually seen. The overlying corneal epithelium may breakdown and can lead to ulceration and stromal thinning where if left untreated, may result in corneal perforation (Pakrou et al., 2006). In GPA, corneal melt has been reported to occur in the earlier stages of the disease and can progress quickly, resulting in visual loss (Galor and Thorne, 2007).

1.6.11 **Eyelid, Adnexal and Conjunctiva Manifestation of GPA**

Eyelid involvement in GPA is rare. Some of the lid manifestations of GPA include ptosis and lid granulomas, and in severe cases, lid destruction. It has been suggested that the 'yellow-lid sign', which is described as resembling florid xanthelasma despite the patient having normal lipid metabolism (Dharmasena et al., 2009), when associated with orbital inflammation, is an indicator to the clinical diagnosis of GPA (Tullo et al., 1995).(Figure 1.8) As for adnexal involvement, dacroadenitis and lacrimal gland enlargements are recognised clinical features of GPA which may cause ocular sicca syndrome i.e. dry eyes (Khanna and Shrivastava, 2011).

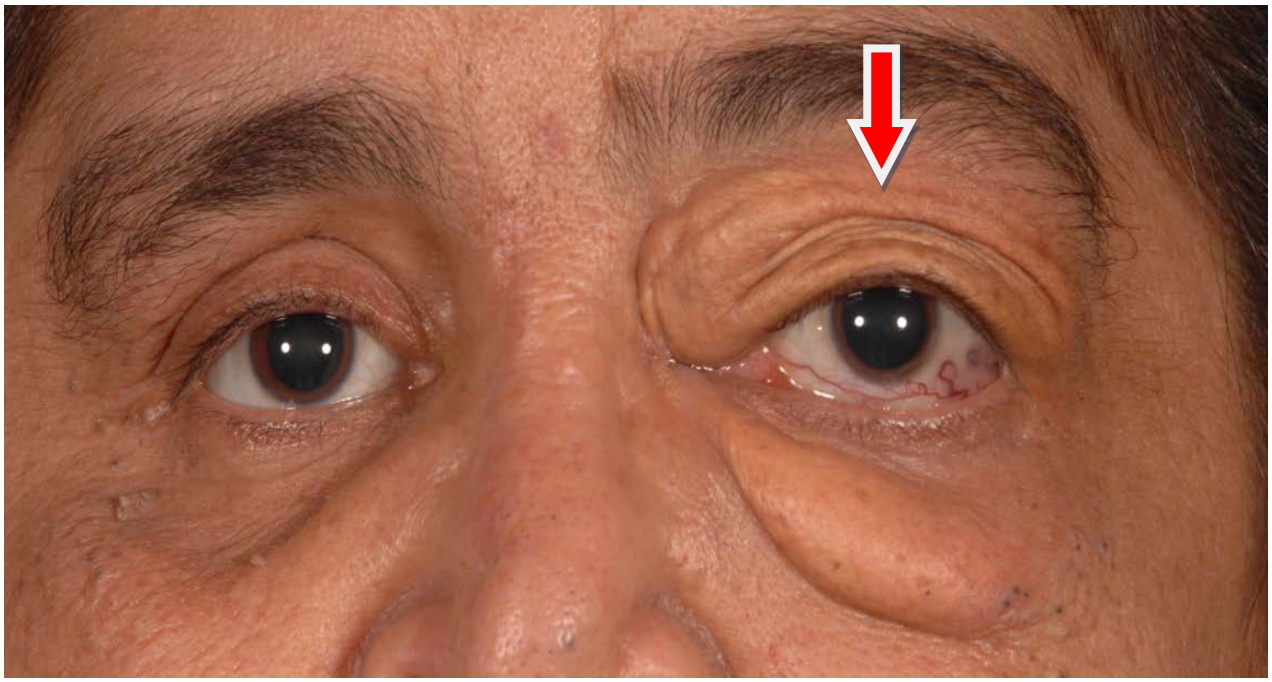


Figure 1.10: Left "yellow-lid sign"(arrow) together with proptosis orbital GPA together

Nasolacrimal disease and nasolacrimal blockage in GPA may lead to the development of dacrocystitis and epiphora. This can occur either as a consequence of inflammatory

spread from the adjacent sinonasal inflammation or a direct consequence of focal GPA inflammation (Tarabishy et al., 2010)(Pakrou et al., 2006).Conjunctiva involvement in the form of ulcerative and necrotic conjunctivitis can occur in up to 16% of patients with GPA (Robinson et al., 2003) and can result in marked cicatricial changes of the conjunctiva (Tarabishy et al., 2010).

1.6.12 **Uveitis, Retina and Choroid Manifestation of GPA**

Uveitis is not a common manifestation of GPA, occurring only in 3% of patients (Bullen et al., 1983). More often, uveitis in GPA is observed to occur concurrently with scleritis and is termed sclerouveitis. This condition is said to most likely carry a poorer ocular prognosis (Pakrou et al., 2006). Retinal and choroid involvement in GPA is also uncommon and can present as retinitis, chorioretinitis, macular oedema, exudative retinal detachment or retinal necrosis (Pakrou et al., 2006). Retinal vein occlusion has also been reported in patients with GPA and is thought to occur due to compression by tissue granulomas to the external vascular wall (Wang et al., 2006). Central retinal artery occlusion (CRAO) in GPA can occur bilaterally and simultaneously. In these cases, the occlusion is thought to mainly be caused by vasculitis. Despite having very poor vision at presentation, prompt treatment of the inflammation may improve the visual prognosis in these patients (Morell-Dubois et al., 2007)(Costello et al., 2005)(Wong et al., 2002). Other reported rare manifestations of GPA in the retina and choroid include vitreous haemorrhage from chorioretinal and ciliary body granulomas (Kamei et al., 2001), acute multifocal placoid pigment epitheliopathy(Chiquet et al., 1999) and sclero-choroidal granulomas mimicking uveal melanoma (Janknecht et al., 1995).

1.7 Treatment Options

1.7.1 General Treatment Regimes

GPA, if left untreated, is a fatal disease with a mean survival period of 5 months for only 50% of patients after diagnosis and 80% would die within a year, mainly from renal disease (Hoffman et al., 1992). Treatment with corticosteroid alone only gives a moderate increase in the mean survival period to 12 months. Fortunately, with increased awareness of the disease and recent improved treatment regimens, severe morbidity and mortality from GPA is becoming less frequent (Holle et al., 2010b).Currently, the combination of corticosteroid with either cyclophosphamide, azathioprine or methotrexate, has shown to achieve disease remission and improve survival, where survival rates with the combination therapy increasing to 95% at 5 year follow up and up to 80% after 10 years. The treatment of GPA in general can be divided into two phases; (1) remission induction by the use of intensive immunosuppressive therapy, and (2) maintenance therapy with less intensive immunosuppressive regimes.

1.7.2 Remission Induction

In 2007, the EUVAS group devised a system of disease sub-groupings for AAVs, based on the severity of the presentation, to guide therapy. Currently, the treatment for these diseases is based on assessing the patient's clinical severity and disease extent and subdividing the disease into three groups: (1) localised and/or early, (2) generalised disease with threatened organ involvement and (3) severe/life threatening disease (Lapraik et al., 2007).

Today, the use of cyclophosphamide (CYC) and corticosteroid (CS) (1mg/kg/day) is recommended for generalised/systemic GPA. However, due to its well recognised serious adverse effects, such as amenorrhoea, infertility, cystitis, haematuria and transient alopecia, the management of GPA is moving away from the use of CYC(Faurschou et al., 2008)(White and Lynch, 2006)(Fervenza, 2010). New treatment regimens with biologics, such as Rituximab (RTX), a monoclonal anti CD20 B cell antibody that depletes B cells, are gaining popularity as the first line of treatment in the management of GPA. In fact, RTX is now frequently used as an alternative treatment regimen for GPA instead of the standard CYC induction protocol recommended by EUVAS. Moreover, many reports have shown RTX to be as effective in the treatment of AAV as CYC(Fervenza, 2010)(Stone et al., 2010)(Jones et al., 2010).

1.7.3 **Remission induction in limited or early GPA**

In limited or early GPA, remission induction has been shown to be successful with the combination of methotrexate (MTX) (20-25mg per week) with CS (1mg/kg/day and gradually tapered) as CYC and prednisolone, thus avoiding the possible adverse effects of CYC in these patients (NORAM study)(De Groot et al., 2005)(Mukhtyar et al., 2009). However, in patients with extensive disease and pulmonary involvement, MTX is less effective in achieving remission compared to CYC and had a higher relapse rate in this group. Nevertheless, for cases of limited GPA, MTX is still widely considered to be the drug of choice for such patients.

1.7.4 **Remission induction in severe or life threatening GPA**

In severe or life threatening GPA (AAV with renal or other vital organ failure and serum creatinine> 500micromol/l), treatment recommendation by EUVAS includes the use of CYC (IV or pulse) and steroid with adjuvant plasma exchange (plasmapheresis; PE). Therapeutic PE involves an extracorporeal blood purification technique that aims to remove large molecular weight substances from the plasma such as immune complexes and pathogenic autoantibodies which may be involved in disease development. This will subsequently reduce tissue damage and promote reversal of the pathological process (Al bshabshe et al., 2010). Therapeutic PE has been used to induce disease remission in severe renal and pulmonary vasculitic diseases including GPA. In GPA patients with pulmonary renal syndrome, PE together with immunosuppressive treatment has also been shown to be successful (Al bshabshe et al., 2010).

Anti-TNF-α agents such as infliximab and etanercept have also been extensively studied for their potential role as remission inducing therapies for vasculitic diseases. Infliximab, a chimeric monoclonal antibody directed against TNF alpha was shown to be effective at inducing remission in 88% of patients with AAV (Booth et al., 2004). Other studies have also reported infliximab to be a potentially useful and safe rescue treatment for patients with refractory systemic necrotizing vasculitides(Josselin et al., 2008). Despite the positive report outcomes, the safety profile of infliximab is still questionable and further study is required.

In contrast, the Wegener's Granulomatosis Etanercept Trial (WGET) unexpectedly found that etanercept was ineffective for the maintenance of remission in patients with Wegener's granulomatosis. In addition, eternecept was also found to be associated with a high rate of treatment related complications, including solid organ tumours(Seo et al., 2005) where long-term follow up of these patients showed that the incidence of solid

malignancy remained increased during long-term follow-up. Although etanercept exposure during the trial could not solely be attributed to the development of this complication, anti-TNF therapy with etanercept do appear to further increase the risk of malignancy in patients with GPA who are treated with cytotoxic therapy and should be avoided for such patients (Silva et al., 2011).

1.7.5 **Maintenance Therapy**

In AAV patients, maintenance therapy following disease remission is recommended as there is a high tendency of occurrence for disease relapse. MTX and Azathioprine (AZA) have both been shown to be as effective as CYC as a maintenance therapy in GPA, thus the duration of exposure to CYC may be safely reduced (Jayne et al.).

In limited GPA, like orbital GPA, it has been shown that the combination of MTX and corticosteroid also achieves remission maintenance rates of around 70% (De Groot et al., 2005). Similarly, mycophenolate mofetil (MMF) has also been demonstrated to achieve remission maintenance and moreover, it is a well-tolerated drug. Unexpectedly however, MMF was found to be less effective than AZA in maintaining disease remission among AAV patients in one study (Hiemstra et al., 2010). Nevertheless, all three treatments have a more favourable adverse effect profile than CYC, thus are a much preferred choice for remission maintenance.

Lefonimide, a pyrimidine synthesis inhibitor that targets T cells by inhibiting the mitochondrial enzyme dihydro-orotatedehydrogenase thus limiting pyrimidine synthesis, is another treatment option. It has also been reported to be an effective alternative immunosuppressive drug for remission maintenance in GPA (Henes et al., 2011)(Metzler et al., 2007).However, there are reports of increased frequency of adverse events (Metzler

et al., 2007).Relapse rates have also been noted to remain high(Villa-Forte et al., 2007) especially in patients who are still ANCA positive at the time of remission. Hence, it is recommended that treatment is maintained for up to 12 months post remission where continued close follow-up of patients during this period is mandatory.

It has been postulated that bacterial and viral respiratory tract infections can trigger relapses in patients with PR3-positive vasculitis. Reports have shown that treatment with co-trimoxazole may be beneficial as this antibiotic would eliminate the offending microbe and as a result, halts the initiating stimulus in these patients. In previous studies, treatment with co-trimoxazole has been shown to be effective in reducing the incidence of relapses in patients with GPA in remission (Zycinska et al., 2009)(Stegeman et al., 1996). In cases of persistent endonasal activity of GPA together with S. aureus carriage, treatment with co-trimoxazole should also be considered (Kallenberg, 2011b). Co-trimoxazole was also recommended by both the EULAR (Mukhtyar et al., 2009) and the British Society for Rheumatology (BSR), and British Health Professionals in Rheumatology(BPHR) guidelines (Lapraik et al., 2007) as prophylaxis against pneumocystis jerovicii infection in patients with WG/GPA.

1.7.6 **Other Treatment Regimes**

15-Desoxyspergualin, a synthetic derivative of spergualin, which is a protein from Bacillus laterosporus that is capable of preventing T-cell and B-cell maturation, has been used with some success in refractory GPA cases (Schmitt et al., 2005). It may offer a safer alternative to CYC for induction therapy, but is not yet supported for routine clinical use. Anti-CD52 therapy (alemtuzumab, CAMPATH-1H), a humanized monoclonal antibody to CD52, has also been shown to have anti-lymphocyte activity and has been reported to

induce remission in AAV. However, relapses and adverse events are common, thus its use in GPA is considered experimental (Walsh et al., 2008). Anti T-cell antibodies such as anti-thymocyte globulins causes rapid depletion of T lymphocytes and have been investigated for use in refractory GPA (Schmitt et al., 2004). Other upcoming potential treatment options for AAV in the future include hematopoietic stem cell transplant, intravenous high dose azathioprine, intravenous immunoglobulins and abatacept.

1.8 Management of Ocular Manifestation of GPA

1.8.1 Medical Treatment for Ocular GPA

In general, the treatment of ocular involvement in GPA requires management of the underlying systemic disease with corticosteroids and cytotoxic agents. Although the disease may be confined to the eye, collaboration between ophthalmologists and physicians for thorough evaluation, investigation, management and future monitoring is essential. This is because the ocular presentation may be the initial presentation of GPA and could eventually progress to involve other organs over time. In cases where clinical evidence of GPA is strong, such as eye symptoms with corresponding sinus or upper respiratory characteristics of GPA, necrotizing scleritis particularly with corneal infiltrates or concurrent uveitis, or bilateral ocular inflammation, treatment should be advocated even if serology testing for ANCA is negative.

Although ocular GPA usually does not respond to topical agents, topical treatment can be considered in milder presentations such as episcleritis or mild anterior uveitis. Nevertheless, in rare cases of uveitis, local injection of CS may also be advocated. Non-steroidal anti-inflammatory drugs (NSAIDS) can be administered in cases of non-

necrotising anterior scleritis. In the more severe form of ocular GPA such as necrotizing scleritis, bilateral ocular involvement, orbital and adnexal presentation, posterior scleritis and refractory cases, aggressive immunosuppressive treatment with CYC and CS are required to ensure adequate control. RTX has been reported to be effective in treating various ocular manifestations of GPA such as optic nerve infiltration(Shunmugam et al., 2011), peripheral ulcerative keratitis (Huerva et al., 2010), optic neuritis (Huchzermeyer et al., 2010) and relapsing necrotizing scleritis(Onal et al., 2008a)(Onal et al., 2008b), although remission may take up to seven months to occur (Taylor et al., 2009).

1.8.2 **Surgical Intervention for Orbital GPA**

Surgical procedures in the management of orbital GPA are mostly performed for diagnostic purposes i.e. orbital biopsy. In cases of severe ocular proptosis secondary to GPA, conventional medical therapy is often unsuccessful. In these cases where proptosis can be associated with pain and optic nerve compression and are refractory to medical therapy, surgical orbital decompression should be considered (Hernández-Rodríguez et al., 2010). Prompt diagnosis and surgical decompression following acute visual deterioration can result in good visual outcome (Fishman et al., 2008). Despite this, related ocular symptoms such as diplopia may still be difficult to resolve, even when orbital decompression is performed. Dacryocystorhinostomy (DCR) has been shown to be effective in restoring nasolacrimal duct NLD function in cases of nasolacrimal obstruction secondary to GPA. DCR performed during disease remission and low ANCA titres usually result in the best outcomes.

1.9 Problem statement

1.9.1 The Diagnostic Challenges in Orbital GPA

The diagnosis of GPA, particularly the limited form such as orbital GPA, is challenging and entails a combination of clinical, serological, radiological and histological features. In general, clinicians require a heightened level of clinical suspicion to make judgements, particularly as the severity of disease in GPA may range from a non-specific inflammatory picture involving only one site or organ, to fulminant multi-organ vasculitis that can lead to death (White and Lynch, 2006).

Classification criteria and definitions for AAV including GPA, for the use of epidemiological studies have been proposed by the American College of Rheumatology (ACR) and the International Consensus Conference at Chapel Hill (CHCC). However, it has been noted that these proposed classifications are rather limited, mainly because they do not include the presence of ANCA as a diagnostic criterion (Leavitt et al., 1990). The European League Against Rheumatism/European Vasculitis Study (EULAR/EUVAS) group further developed the criteria for the classification of AAV which not only combined the recommendations from the ACR and CHCC but also included surrogate biomarkers (Hellish et al., 2007).In 2007, a stepwise algorithm was then developed by consensus between a group of doctors interested in the epidemiology of vasculitis, for the classification of AAV and polyarteritis nodosa (PAN) (Watts et al., 2007). This includes a four step algorithm incorporating both the ACR and CHCC systems that has been shown to be effective in classifying patients into a single category(Liu et al., 2008)(Kallenberg, 2008).

Similarly, in paediatrics, a EULAR/Paediatric Rheumatology European Society (EULAR/PReS) working group using a Delphi technique had developed the classification

criteria for childhood vasculitis(Ozen et al., 2006).An agreement was reached to classify childhood vasculitis according to vessel size, with small-vessel diseases subdivided into granulomatous and non-granulomatous. The ACR criteria for GPA were modified to include two new criteria: (1) the presence of subglottic, tracheal or endobronchial stenosis, and (2) the presence of high levels of PR3-ANCA or positive c-ANCA by indirect immunofluorescence (Watts and Scott, 2009).

Nevertheless, it is important to acknowledge that all these classification criteria were developed mainly for clinical studies, not for diagnostic purposes and have been shown to have their own individual limitations (Rao et al., 1998)(Bruce and Bell, 1997). Therefore, these guidelines should not serve as a definitive tool for clinical diagnosis (Watts and Scott, 2009). In addition, in the limited form of GPA, like ocular GPA, classical features of the disease are often lacking and may not fit any classification or definition. Moreover, ANCA presence and level is a poor indicator for diagnosis and disease activity in the limited form of GPA being positive only in up to 65% of patients (Tarabishy et al., 2010)(Lamprecht and Gross, 2004). As a result, the diagnosis of limited GPA may be missed or delayed and only detected once disease progresses to a more severe systemic form (Hoffman et al., 1992)(White and Lynch, 2006).

1.9.2 **Clinical Presentation**

The clinical presentation of orbital GPA, as described previously, often overlaps with the clinical presentation of other orbital inflammatory conditions thus poses difficulty in establishing a conclusive diagnosis. The manifestation of orbital inflammatory disorders (OID) is vast. The differential diagnosis of GPA can be generally divided into autoimmune, inflammatory, infectious and neoplastic causes. In particular, sarcoidosis and IIOD are the

main differential diagnosis for GPA. Sarcoidosis shares the same disease process as GPA, and IIOD tend to share very similar clinical presentation with GPA as well as possibly also presenting a granulomatous inflammatory picture histologically. Other usual main differential diagnoses considered with GPA include TED, IgG4 and orbital cellulitis, among others.

1.9.2.1 Orbital sarcoidosis

Sarcoidosis is also a multisystem granulomatous disorder. Similar to GPA, it can manifest systemically or confined locally like in the eye. Pulmonary involvement is the most common manifestation of the disease occurring in 90% of patients, and 15% of these patients may exhibit ocular involvement. Uveitis is the most common ocular presentation in ocular sarcoidosis, with iridocyclitis being acutely and frequently associated with systemic sarcoidosis. Iris nodules and keratic precipitates on corneal endothelium can be seen in the anterior segment. In the posterior segment of the eye, vitreous aggregations can be seen and nodular granuloma may be present on the optic disc, retina and choroid. Nodular granuloma along retina venules, often described to resemble candle-wax dripping, is a classic sign of ocular sarcoidosis. Apart from the uvea and retina, any ocular tissues can be affected by sarcoidosis, including the orbit and adnexa. Granuloma formation with inflammation in the orbits can occur in patients with orbital sarcoidosis where presentations such as proptosis, ocular motility dysfunction and ocular pain resemble orbital presentations of GPA. Sarcoidosis can also affect the lacrimal gland causing severe discomfort as well as upper lid swelling and inflammation, also similar to GPA. The presence of eyelid granuloma and conjunctiva nodules can be useful for tissue biopsy purposes in these cases.

1.9.2.2 IIOD

IIOD is a space occupying inflammatory disorder that stimulates other inflammatory disorders such as GPA, TED, sarcoidosis as well as neoplasm but has no identifiable cause. It is the main differential diagnosis for nearly all OIDs particularly GPA. Clinically, patients present with acute pain and redness and can present with diplopia when the extraocular muscles are involved. IIOD can also affect the lacrimal glands causing severe acute dacryoadenitis. Fibrosis is the predominant feature of the late stage of the disease where ocular motility may be affected permanently. IIOD responds promptly with corticosteroids.

1.9.2.3 Thyroid eye disease (TED)

TED is the most common cause of systemic related orbital inflammation and may affect one eye or both eyes at the same time(Kim et al., 2010). As with GPA, TED may present proptosis, poor vision and red eye but other typical features of ocular TED such as lid lag and lid retraction together with systemic symptoms of thyroid dysfunction may help in distinguishing it from other OIDs. Raised anti thyroid antibodies and an abnormal thyroid function test may also assist in the diagnosis of TED, although normal levels do not exclude it.

1.9.2.4 IgG4 related disease

IgG4-related disease is a systemic lymphoproliferative disorder. Affected organs in this disease show fibrotic and sclerotic changes with hyper-IgG4-gamma-globulinaemia and

IgG4-producing plasma cell expansion. In the orbit, it frequently affects the lacrimal gland where common presentations of the orbit include bilateral symmetrical lacrimal gland swelling and dacryoadenitis. Other ocular symptoms similar to ocular GPA include proptosis and eyelid or periocular swelling. The diagnosis of IgG4-related disease is made by performing blood investigations as well as lacrimal gland biopsy. Serologically, patients will have high levels of serum IgG4 (>135mg/L) and lacrimal biopsy tissues will show marked IgG4 positive plasmacyte infiltration (>40% IgG4-positive/IgG-positive cells in five high-power fields) (Masaki et al., 2011)(Plaza et al., 2011).

1.9.2.5 Orbital cellulitis

Infective causes such as orbital cellulitis from bacterial or fungal infections is another differential diagnosis of OID. It is particularly important to exclude this diagnosis prior to commencement of immunosuppressive treatment, especially when there is a history of trauma to the sinuses or dental infection or intervention (Tarabishy et al., 2010). Patients with orbital cellulitis frequently present symptoms of infection such as fever, tachycardia and discomfort. Blood investigations typically show leucocytosis and performing blood cultures may be useful in identifying the infective agent and excluding the diagnosis of other OIDs.

1.9.2.6 Other orbital diseases

Apart from GPA, other systemic inflammatory disorders with orbital presentations include Churg-Strauss syndrome, Tolosa-Hunt syndrome and Erdheim-Chester syndrome. Rheumatoid arthritis may present episcleritis, scleritis and peripheral ulcerative keratitis.

Rhabdomyosarcoma and lymphoma are examples of neoplastic disorders that may present proptosis similar to GPA. Orbital tumour metastasis from a remote primary tumour should also be considered (Rizvi et al., 2010)(Plaza et al., 2011).

1.9.3 **Serology**

In current clinical practice, ANCA detection is used as one of the tools for the diagnosis of GPA. However, the absence of ANCA does not preclude this diagnosis. In systemic GPA, c-ANCA is found in about 74-90% of patients, and in 80-95% of these patients the antibody is directed against PR3 (Tarabishy et al., 2010)(Lamprecht and Gross, 2004)(Tsiveriotis et al., 2011)(Wiik, 2002). It is reported that the diagnostic sensitivity is 91% for c-ANCA and 63% for PR3-ANCA with a specificity of 99% for both for the diagnosis of active GPA (27 of 319 patients) (Hagemo et al., 2002)(Taylor et al., 2007)

ANCA titre has also been suggested to act as an indicator of the disease activity, such as predicting disease relapses. Nonetheless, ANCA titre is not entirely reliable and should not solely be relied upon in the management of GPA patients as it has been shown thatPR3-ANCA also increases in patients with GPA upon reduction or withdrawal of immunosuppression without any relapse (Finkielman et al., 2007)(Rasmussen et al., 2013). Furthermore, in the limited form of GPA, ANCA is only found to be positive in 50-65% of patients. Therefore, ANCA presence and level is a poor indicator for diagnosis and disease activity (Tarabishy et al., 2010)(Lamprecht and Gross, 2007).

1.9.4 **Radiology**

Radiographic investigations are used regularly in the investigation of orbital masses. Radiological features of orbital and sinus involvement are demonstrable in approximately 70% of patients with clinical orbital involvement. Imaging with CT and/or MRI is of value in the diagnosis of orbital GPA to detect orbital mass lesions, to observe the extent of the orbital mass within the orbit and to delineate involvement of adjacent structures (Simmons et al., 1987)(Provenzale et al., 1996)(O'Sullivan et al., 1992).

Orbital mass associated with sinonasal changes and paranasal bony erosions on imaging are features highly suggestive of orbital GPA. The presence of bony erosions in GPA is reported to be independent of ANCA status and can occur in both systemic and localised form of GPA (Tan et al., 2014).

1.9.4.1 Computer Tomography Scan (CT scan)

CT scanning has better ability to assess sinus opacification and bone invasion, which is a common finding in GPA (Lohrmann et al., 2006). Classically, appearances on CT are of an ill-defined soft tissue mass which may wrap the globe, concealing the optic nerve and extraocular muscles, with associated local bony destruction. (Figure 1.9)Orbital lesions on unenhanced CT images typically appear homogeneous and isodense relative to the extraocular muscles. On the other hand, the contrast-enhanced CT appearance of orbital lesions is that of a moderately heterogenous mass that is isodense or mildly hyperdense relative to nasal mucosa. CT scanning with contrast, nevertheless, can show a wide range of enhancement characteristics with extreme diversity of enhancement values (Allen and Harvey, 2007). Recently, new techniques using 18F-fluorodeoxyglucose positron emission

tomography/computed tomography (FDG PET/CT) has been shown to be a feasible modality for evaluating lesion activities, therapeutic monitoring, and follow-up of GPA as well as detecting GPA lesions for the purpose of biopsy (Ito et al., 2012). However, to date there are no radiological features that can make a specific diagnosis for GPA.

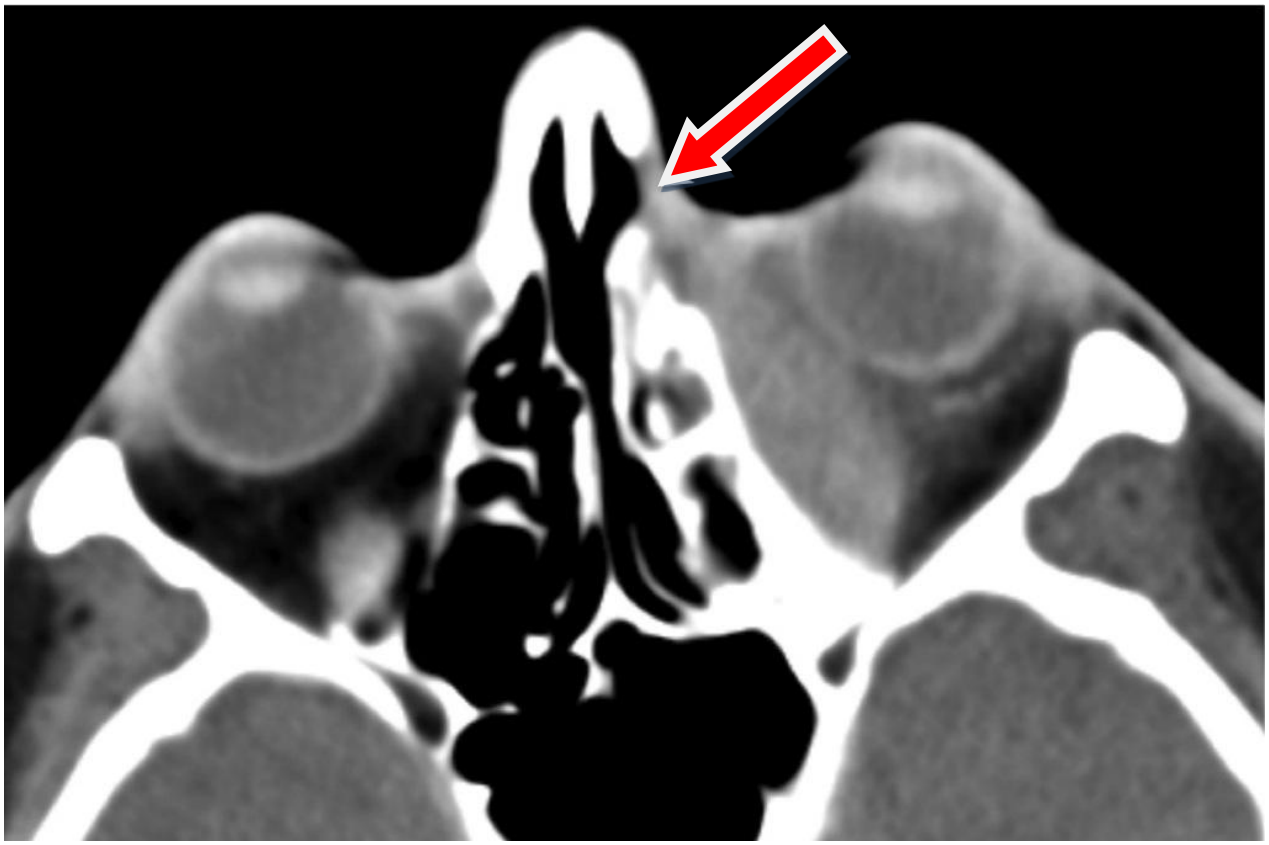


Figure 1.11: Axial CT scan of the orbit in GPA showing ill-defined soft tissue mass in the right orbit wrapping the globe, concealing the optic nerve and extraocular muscles, with associated local bony destruction (arrow).

1.9.4.2 Magnetic Resonance Imaging (MRI) (Figure 1.10)

MRI may be useful in characterising orbital lesions in GPA (Figure 1.10). Granulomas on T1 and T2 weighted images display low-to-intermediate signals and can be distinguished

from the surrounding orbital fat tissues and extraocular muscles (Muhle et al., 1997)(Razek et al.). Nevertheless, it is not possible to differentiate between mucosal inflammation and granulomatous tissue on MRI during the initial inflammatory process of GPA. This is because granulomas can only be distinguished as low-signal-intensity lesions on MRI in the later stage of granulomatous development. On T2 weighted images, a marked decrease in signal is said to be characteristic of GPA. However, this is not pathognomonic for the disease as similar findings can sometimes be seen with idiopathic orbital inflammation, chronic lacrimal gland sarcoidosis and in metastatic melanoma of the orbit (Allen and Harvey, 2007)(Courcoutsakis et al., 1997).

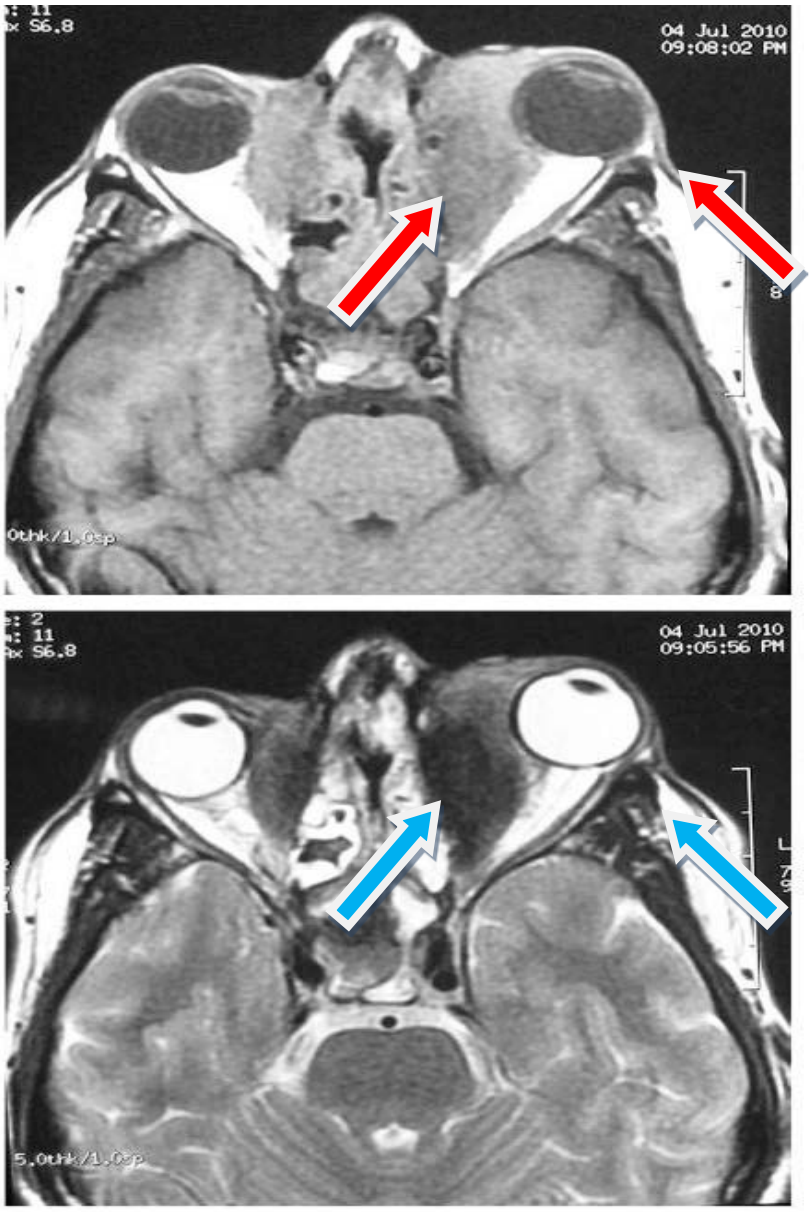


Figure 1.12: MRI Orbital magnetic resonance imaging in GPA showing heterogenous bilateral orbital mass lesions adjacent to the medial walls. The lesions have moderate intensity, similar to muscle on axial T 1-weighted (top, red arrows) and low intensity on axial T 2-weighted images (below, blue arrows)

Injection of dye contrast during MRI is commonly performed to further highlight lesions in the images. In orbital GPA, following injection of dye contrast, varying degrees of image enhancement may be seen (Razek et al.). After gadolinium injection, granulomas may portray homogenous (Provenzale et al., 1996) or heterogeneous signal enhancement, or,

in a minority of cases, no enhancement (Muhle et al., 1997). GPA should therefore be considered as one of the differential diagnosis for patients with low to medium-signal-intensity lesions on T1- and T2-weighted sequences of the nasal cavity, paranasal sinuses and orbits. However, it is noted that the unenhanced, non-fat suppressed T1-weighted sequence is the preferred method for lesion detection in orbital GPA in one study (Courcoutsakis et al., 1997).In addition, bony erosion of the orbit and sinuses mainly seen affecting the ethmoid sinuses, nasal septum and medial walls of the orbit, has also been demonstrated in GPA (Muhle et al., 1997)(Razek et al.).

1.9.5 **Histology**

Histology investigations are important in the management of all OIDs. In GPA, classic histological features of GPA are described from the histology of larger organs affected by the disease such as the lungs and kidneys. The classic pathological features associated with GPA include granulomatous inflammation with involvement of multinucleated giant cells and epithelioid cells seen to predominate; microabcess or fibrinoid necrosis which appear widespread or geographic like, and vasculitis; affecting medium to large size vessels. (Figure 1.11)

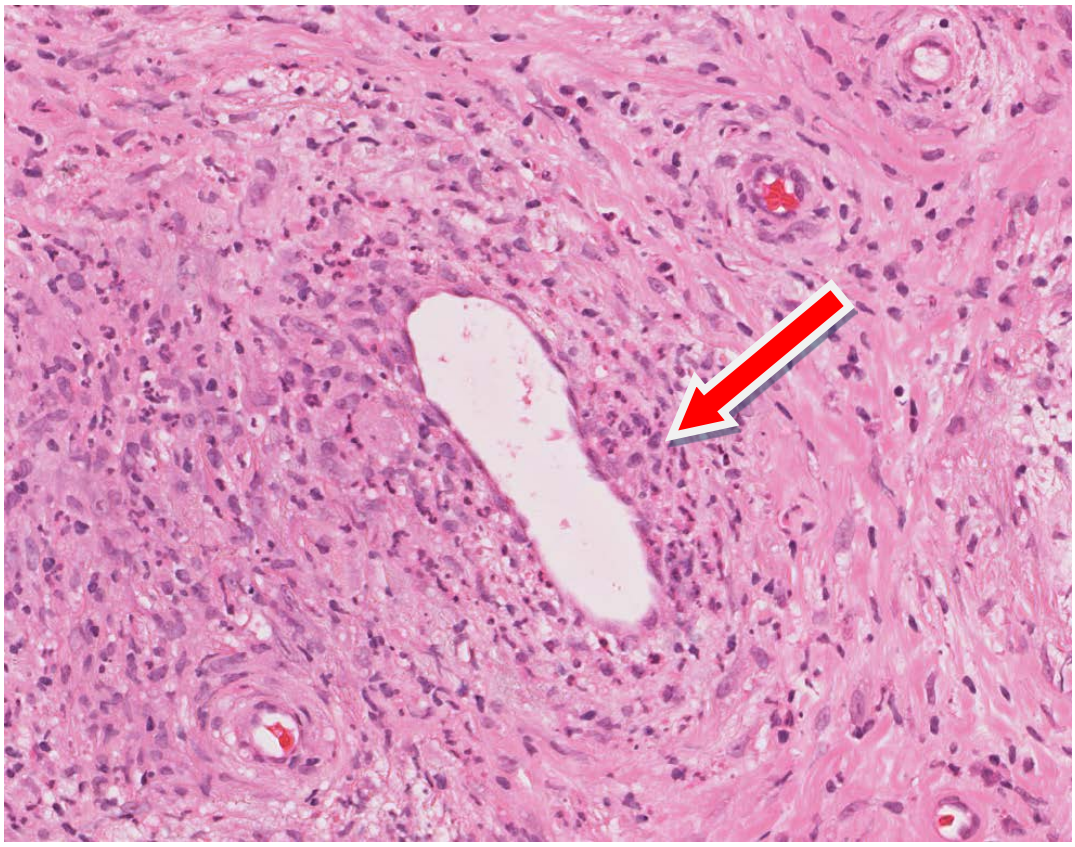


Figure 1.13: Histology of vasculitis (yellow arrow) seen in GPA tissue biopsy. There are also presence of surrounding epithelioid cells with eosinophils, neutrophils and lymphocytes.

Yet, despite the ongoing active disease, these histological features of GPA are not always seen in all biopsy material. This is possibly truer in the case of the limited form of GPA, particularly the ocular GPA as tissue biopsy specimens are often too small to capture the whole pathologic picture, or the inflammatory process is too mild to mount a typical tissue cellular response. In one report, it is stated that classic histological features for GPA are only present in 25% of biopsies in known GPA patients (Raynaud et al., 2005). The appearance of granulomatous inflammation in tissue biopsies may also represent other various OIDs such as sarcoidosis and IIOD. Although it is said that the findings of granulomatous inflammation with vasculitis generally points out to the diagnosis of GPA, this is probably easier seen in larger tissue samples like the lungs.

1.10 Side Effects and Long-term Outcome

Current improved treatment regimens have led to an overall improved survival and morbidity rates in patients with GPA. In addition, the toxic profiles of these new drugs also seem to be more favourable and are better tolerated by patients compared to earlier conventional standard treatment. The 5 year survival probability among GPA patients is now reported to be within 65-75% in most studies. Mortality is mainly attributed to infection, active vasculitis and renal failure where the risk of death appear to occur highest in the first year of disease, especially among patients less than 65 years of age (Flossmann et al., 2011).

In the eye, up to 17% of cases of patients with ocular GPA can end up with significant visual problems. Particularly in orbital GPA, orbital masses are found to represent a refractory form of GPA; generally unresponsive to immunosuppressants. Patients with orbital GPA are thus prone to develop significant ocular complications with 20-50% of patients developing severe visual loss (i.e. 6/60 or less) (Taylor et al., 2007)(Holle et al., 2013b).Visual loss in GPA is largely caused as a result of corneascleral damage, macular oedema, optic nerve compression from orbital mass or vascular occlusion. Due to this, prompt disease recognition, management and regular follow up by an ophthalmologist are therefore mandatory.

1.11 Area of Research Interest

In general, GPA is a challenging disease to diagnose and manage. This is more so for ocular GPA, given its overlapping clinical presentations with other ocular inflammatory

disorders, low percentage of ANCA positivity and the lack of classic histological findings in biopsies, hence, making it indistinguishable from other orbital inflammatory diseases. Due to this, the diagnosis of ocular GPA is often delayed and consequently, appropriate treatment is not advocated early. The diagnosis is often only established once severe ophthalmic damage, mostly irreversible or permanent vision reduction or loss, has occurred. In some cases, diagnosis may only be clear once renal involvement is apparent or other organ damage has developed.

Further understanding in the immune response involved in vasculitides may help distinguish GPA from other AAV and OIDs. Currently, there are no tissue biomarkers specific for GPA in ocular, nasal and renal biopsies to enable more accurate diagnosis in the early localised stage of the disease and to identify individuals who are at most risk of developing severe organ failure. However, recent successful therapeutic agent for GPA like RTX, which specifically targets CD20 B cells, brings further light to the pathogenesis of GPA. Identifying tissue markers or cellular patterns in tissues biopsies that are specific for GPA and that can be easily identified in a regular pathology laboratory would be of great value to aid and improve diagnosis. There is also little understanding of how current treatment regimens control cellular and humoral factors involved in the pathogenesis and progression of GPA. Identifying a key feature in tissue biopsy; either cellular or tissue change, which can predict disease remission and disease extension or relapse would also be of great benefit. This key feature could lead to a risk stratification and provide aid in formulating a rational treatment approach for GPA, especially in the early stages of the disease course.

1.12 Research Hypotheses

1) There are cells or tissue changes that are found only or more in orbital biopsies of GPA compared to the biopsies of OIDs. These specific cell or tissue changes could then be used as an indication for the diagnosis of orbital GPA in the absence of typical histological features of GPA and serum ANCA.

2) There are cells or tissue changes that are found only or more in orbital biopsies of GPA patients who progress to generalised GPA compared to orbital GPA that remain localised. These specific cell or tissue changes could then be used as a predictor for the clinical outcome of orbital GPA.

1.13 Aims of the study

The aims of our study are as follows:

1. To identify cell types, markers and tissue changes present in orbital tissue biopsies, which are indicative of the diagnosis of orbital GPA despite the absence of classical histological features and a negative serum ANCA.

2. To identify tissue features in orbital biopsies which correlate with clinical outcome and in particular, to determine whether there are tissue biomarkers that could predict disease progression or extension.

2 Chapter 2: Long-term Outcome of Ocular GPA

2.1 Overview

The eye and the orbit can be affected by GPA in up to 50-60% of patients (Joshi et al., 2009)(Pakrou et al., 2006). In 8-16% of patients, the ocular manifestation in GPA is the primary presentation of the disease (Pakrou et al., 2006) where in the remaining majority, the eye is affected due to disease spread either from adjacent affected structures or as part of the overall systemic manifestation of the GPA (Taylor et al., 2007).

Until recently, GPA is mainly described as an inflammatory disorder with multi-systemic organ involvement and can be life threatening when major organs are affected. In patients with renal GPA, long term outcome of these patients shows a poor survival rate; 41% death within six years follow up, and renal function outcome despite intravenous cyclophosphamide treatment (Gottenberg et al., 2007). Survival rates in ANCA positive kidney transplant patients however were better. Nevertheless, it is noted that patients with concurrent ANCA positivity and GPA faired less better in the long run, as the disease relapse rate was higher in this group compared to patients who were ANCA positive but with no GPA (Marco et al., 2013). In patients with ANCA positive pulmonary vasculitis, patients with GPA i.e. PR3-ANCA positive, were seen to develop severe alveoloar haemorrhages, more commonly occurring in nearly 70% of patients with ANCA associated vasculitis, compared to MPO-ANCA patients. The development of alveolar haemorrhages was also reported to have a strong correlation with concurrent renal vasculitis that can lead to renal failure and mortality, particularly in the older age group (Hruskova et al., 2013).

In cases of localised GPA, there are currently mixed reviews with regards to the long-term outcome of these patients. The localised forms of GPA are usually considered to represent an early and milder manifestation of the disease, and are thought to have the potential to progress to the aggressive systemic form in due course. Studies in the past have reported

disease progression from localised orbital manifestation of GPA to fulminant systemic disease with renal impairment, particularly when patients were not treated adequately at the initial stage (Choopong et al., 2005)(Power et al., 1995). In contrast, other larger studies which included the monitoring of patients with localised head and neck and pulmonary GPA, have shown that localised GPA did not progress to the systemic form over time and tend to remain localised (Fechner et al., 2002)(Stone, 2003)(Holle et al., 2010a).

GPA with ocular and/or nasal presentation as the first symptom of the disease is easily misdiagnosed (Jiang et al., 2013)(Jiang B 2013), and orbital GPA are still considered to be a rare manifestation of GPA. However, the presence of orbital GPA has been regarded to characterise a refractory course of the disease and is reported to have a high rate of local damage that can result in irreversible ocular damage and function (Holle et al., 2013b)(Holle 2013).

In general, it would be of great benefit to be able to identify GPA in the early stages of the disease or at the stage when the disease may still be confined within non-life threatening organs such as the eye. However, as previously described, the diagnosis of orbital GPA is very challenging. This further causes dilemma, particularly in the management of orbital GPA, as treatment options for GPA often involve drugs with cytotoxic properties and undesirable side effects.

Thus, for the purpose of this study, it was first necessary to look at the clinical features of orbital GPA, to examine the effect of the disease on patients and the outcome after receiving treatment. More importantly, we wanted to identify patients with localised GPA who progressed to the systemic form of the disease in order to better understand the

pattern of the disease progression and in particular, to examine their histology to try and identify potential markers for GPA, within the tissues.

2.2 Aims

The aim of this section of the study was to observe the clinical presentation, disease progression and the long-term clinical outcome of patients with orbital GPA in our centre. We wanted to determine:

1) The clinical features and long-term outcome of ocular GPA in terms of its clinical presentation, patients' past medical histories, ANCA status, course of management and outcome (i.e. a minimum of two years of follow up), which have not been previously reported before.

2) The rate of progression of localised GPA to systemic GPA, and to compare the patients' clinical background with patients who remained as orbital or localised GPA.

2.3 Method

2.3.1 Ethical Approval

Ethical approval for the study was obtained from the Moorfields & Whittington Research Ethics Committee (REC ref. no. 09/H0721/75, LIGS 1023).

2.3.2 **Study Centre and Patients**

This was a retrospective study of all patients who had undergone orbital or adnexal biopsy for orbital inflammatory disease at Moorfields Eye Hospital a period of over 21 years.

All patients who had undergone an orbital or adnexal biopsy between 1988 and 2009 were identified from the UCL/Institute of Ophthalmology Pathology Database. Patient details were entered into an electronic database and each patient was allocated a study number. Patient details were anonymised and the primary key (i.e. patient details with matched study number) was kept in a locked filing cabinet. Patients' case notes were then recalled from the Moorfields Eye Hospital medical record library. A comprehensive review of the clinical notes available was then performed.

2.3.3 **Inclusion Criteria**

The diagnosis of orbital GPA is based on several factors and is generally from a combination of either typical ocular presentation of orbital GPA, previous history of GPA in other organs (particularly adjacent organs such as the nose, radiological investigations), positive serum ANCA or histological features in orbital biopsies.

In our study, it was paramount that we avoid confounding factors for the next stages of the study i.e. during histology analysis of orbital biopsies. Therefore, our patient selections (or inclusion criteria) were very specific. We included patients into the study strictly only if their diagnosis and the clinical management for their orbital disease were not influenced by histology investigations. This means that patients were included only if their orbital diagnosis were established from two or more combinations of the following: (1) the clinical history (e.g. previous diagnosis of GPA affecting other organs); (2) typical disease

presentation and manifestations of the disease (e.g. lid lag in thyroid eye disease (TED), necrotising scleritis in GPA); (3) blood investigations (e.g. ANCA positivity for GPA, raised angiotensin converting enzyme (ACE) in sarcoidosis) or (4) radiological tests (nasal bony erosions in GPA, muscle belly enlargement in TED).

In addition, only patients with a minimum follow up period of two years were included in the study.

2.3.4 **Exclusion Criteria**

Patients were strictly excluded from the study if the diagnosis and management of their orbital problem had been influenced or had only been made based on the histological appearance of their orbital biopsy or had a minimum follow up period of less than two years.

2.3.5 **Clinical Data**

A comprehensive review of clinical notes available was performed to obtain information on patient demographics and clinical diagnosis. In the GPA group, we further looked at the orbital and ocular presentation, orbital structure involved for tissue biopsy, ANCA status, other organ involvement (otolaryngology, cardiac, respiratory, renal and central nervous systems), treatments (medical and surgical), duration of follow up and long-term outcome. We also sent out letters and contacted the general practitioners of the patients to obtain further clarification and information with regards to patients' medical progression and status.

2.4 Definition of Terms

2.4.1 Localised GPA

We defined localised GPA as features of GPA confined to the ocular, upper and/or lower respiratory tract disease without any other systemic involvement, non-ocular organ threatening or life threatening disease.

2.4.2 Systemic GPA

GPA patients who did not fulfil our criteria for localised GPA were classified as general GPA.

2.4.3 Progression of GPA

We defined disease progression as individuals with localised GPA that further developed general GPA from the period of their orbital manifestation.

2.4.4 Newly Diagnosed GPA

This is defined as patients who were never previously diagnosed with GPA (in any organ system) before the orbital biopsy. The diagnosis of GPA was made at the ophthalmology presentation.

2.4.5 Known GPA

This is defined as patients who are known cases of GPA where the diagnosis of GPA was established prior to the ocular presentation.

2.5 Results

2.5.1 Patient Characteristics

A total of 470 patients had been identified via the Moorfields Eye Hospital database and biopsy specimen records, to have undergone orbital biopsy for orbital inflammatory disorders between the years 1980 to 2009. 234 patients fulfilled our inclusion criteria and were therefore included in the study. The majority of patients excluded from the study were those with their orbital diagnosis mainly based on or influenced by the histological appearance of their biopsies. Others were excluded due to incomplete clinical data, lost files or a follow up period of less than two years.

The mean follow up time for the whole group were 47.26 SE 5.051 months (i.e. 3.9 years), ranging from a minimum period of 23.9 months to 12.1 years. The mean age of the whole sample population was 50.5 SE 1.11 (i.e. 12-97) years old. Patients' characteristics in the GPA and non GPA patients are listed in Table 2.1. There was a female preponderance seen in the sample (female = 147, male = 87) with a female to male ratio of 3:2. (Figure 2.1)Both GPA and non GPA groups were similar in age and gender. (Table 2.1)

Table 2.1: Patient characteristics in GPA and non GPA group

	GPA (n=36)	Non GPA (n=198)
Age (mean (range))	50.7 (14-87)	50.4 (12-97)
Gender		
Female (n(%))	21 (58.4%)	127 (64.2%)
Male (n(%))	15 (41.6%)	71 (35.8%)
Female : Male	3:2	3:2

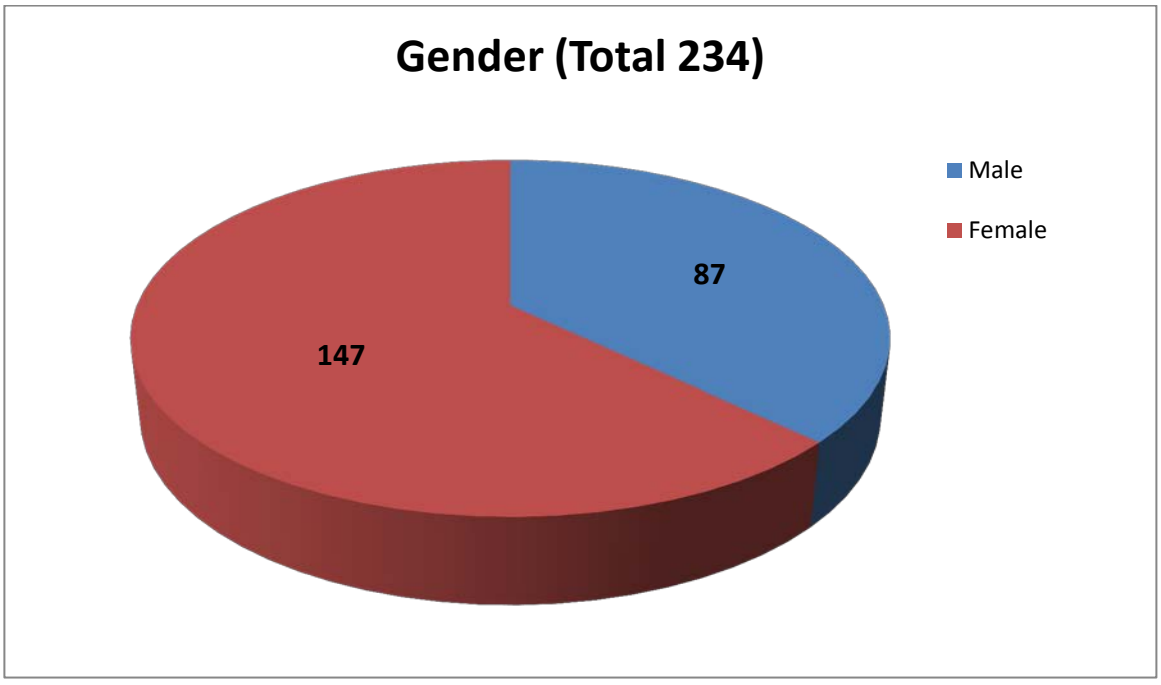


Figure 2.1: Gender distribution of whole sample population

Of the 234 patients, 36 (15.4%) patients fulfilled our criteria for the diagnosis of GPA and 198 were classified as non GPA. The distribution of diseases in the non GPA group included 67 chronic idiopathic inflammation of the orbit (IIOD) (28.6%), 16 lymphoid hyperplasia (6.8%), 14 sarcoidosis (5.6%), three myositis (1.3%), 33 dacroadenitis (14.1%), 12 thyroid eye disease (5.1%) and 54 miscellaneous diseases e.g. eosinophilic angiocentric fibrosis, Churg-Strauss disease, foreign body etc. (23.1%). (Figure 2.2)

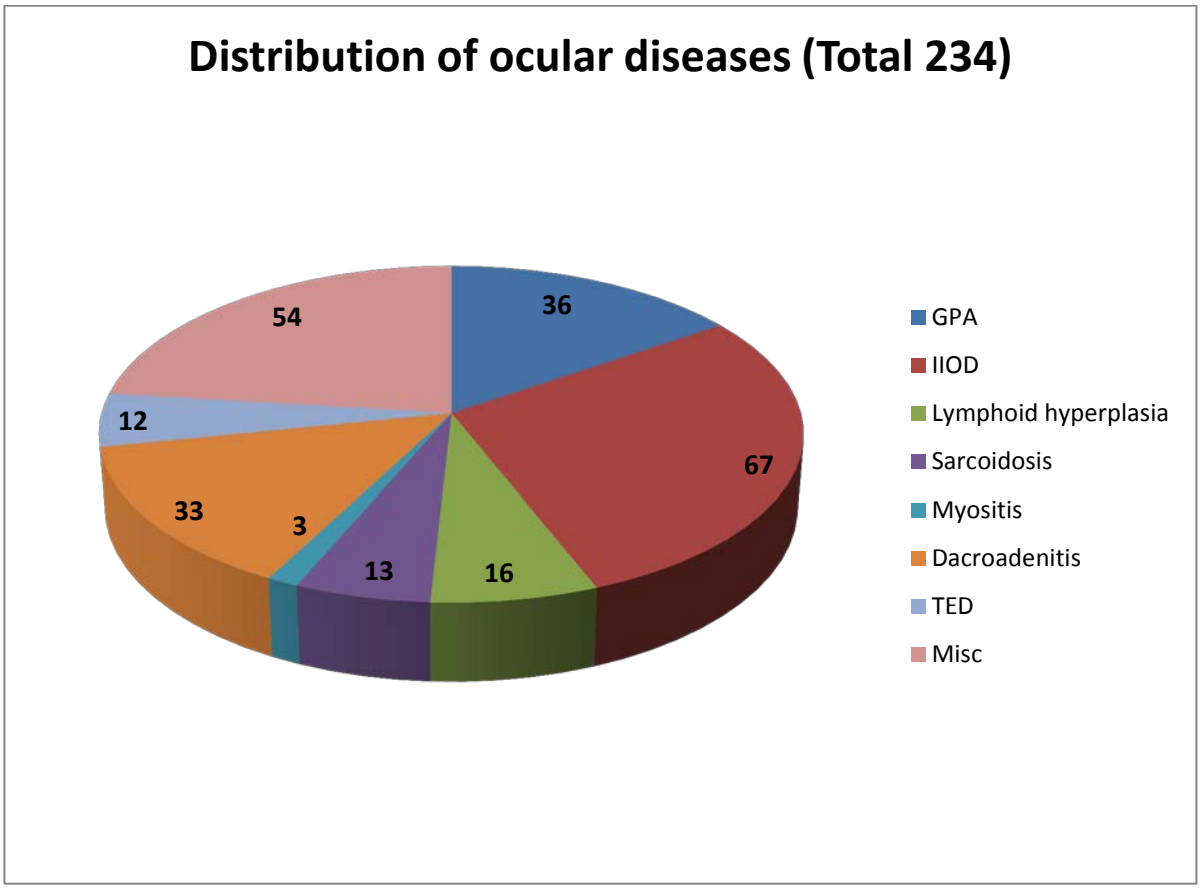


Figure 2.2: Distribution of ocular diseases in sample population

The majority of biopsies in the whole study population were from orbital masses (45.3%). 37% of patients had biopsies taken from the lacrimal gland. The remaining 17.7% of the biopsies were taken from other orbital structures such as nasolacrimal duct/sac, extraocular muscle, sclera and eyelid. (Table 2.2)

Table 2.2: Organ involvement in GPA patients

Number of organs affected by GPA	**Organs involved**	**Total patients**
1 organ	**Limited to orbit only**	**6**
2 organs	**Orbit + sinonasal**	**20**
3 organs	**Total**	**7**
	orbit + sinonasal + lungs	5
	orbit + sinonasal + kidney	1
	orbit + sinonasal + central nervous system	1
4 organs	**Total**	**3**
	orbit + sinonasal + lungs + kidneys	1
	orbit +sinonasal + lungs + cardiovascular	2

2.5.2 **GPA Group**

The patient characteristics and clinical overview in the GPA group are summarised in Figure 2.3,Table 2.2 and Table 2.3.

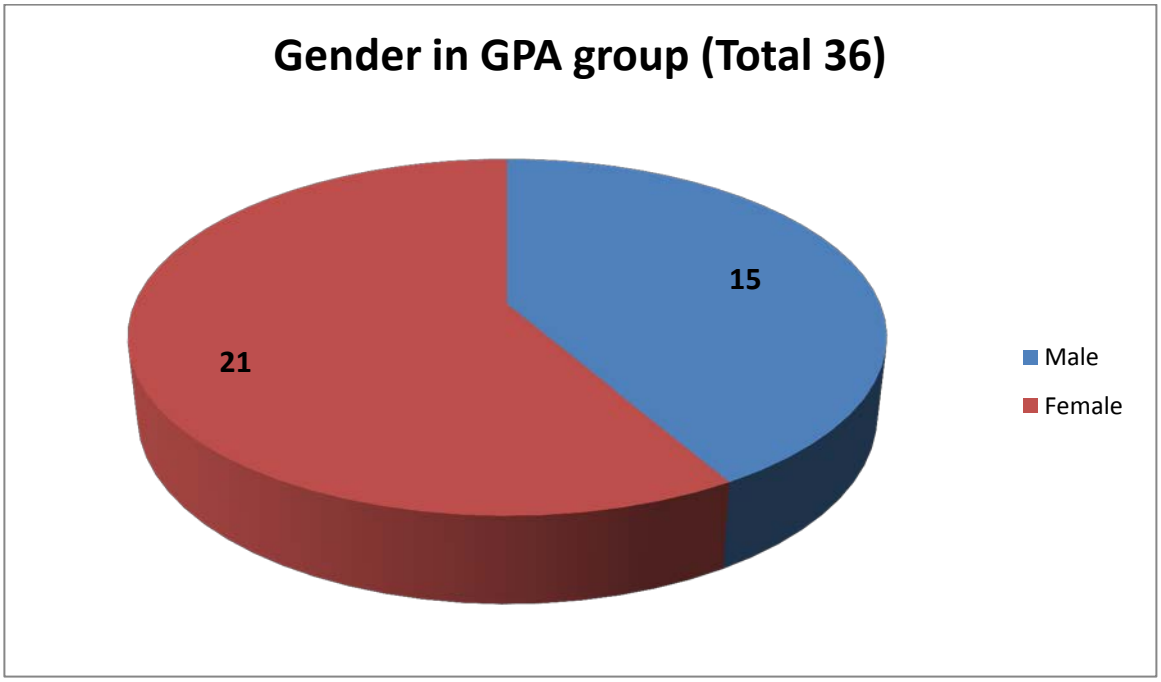


Figure 2.3: Gender distribution in the GPA group.

Table 2.3: Summary of patient characteristics within the GPA group; comparing between newly diagnosed GPA (initial presentation of GPA) and known GPA (recurrent or orbital extension of the disease)

Patient characteristics		Known GPA (n=19) Total (% in sub-group)	Newly diagnosed GPA (n=17) Total(% in sub-group)	Total (n=36) (% of total)
Gender:	Male	8 (42.1%)	7 (41.1%)	15 (41.7%)
	Female	11 (57.9%)	10 (58.8%)	21 (58.3%)
Mean follow up time (months +/-SEM)		28 +/- 3.1	27 +/- 2.2	
Laterality:	Unilateral	18 (94.7%)	16 (94.1%)	34 (94.4%)
	Bilateral	1 (5.3%)	1 (5.9%)	2 (5.6%)
Clinical presentation:	Proptosis	7 (36.8%)	10 (58.8%)	17 (47.2%)
	Lacrimal gland enlargement	3 (15.8%)	8 (47%)	11 (30.5%)
	Lid swelling	7 (36.8%)	7 (41.1%)	14 (38.9%)
	Decrease vision	0	9 (52.9%)	9 (25%)
	Orbital pain	5 (26.3%)	9 (52.9%)	14 (38.9%)
	Red eye	4 (21%)	3 (17.6%)	7 (19.4%)
	Diplopia	7 (36.8%)	7 (41.1%)	14 (38.9%)
	Reduced ocular motility	5 (26.3%)	7 (41.1%)	12 (33.3%)
	Globe displacement	2 (10.5%)	7 (41.1%)	9 (25%)
	Nasolacrimal/sinonasal (epiphora/dacryocystitis)	14 (73.7%)	2 (11.8%)	16 (44.4%)
	Facial numbness/pain	7 (36.8%)	0	7 (19.4%)
PR3-ANCA:	Positive	12 (63.1%)	9 (52.9%)	21 (58.3%)
	Positive on presentation	7 (36.8%)	6 (35.3%)	13 (36.1%)
Structure biopsied:	Orbital mass	6 (31.6%)	15 (88.2%)	21 (58.3%)
	Lacrimal gland	8 (42.1%)	3 (17.6%)	11 (30.5%)
	Nasolarimal duct	11 (57.9%)	2 (11.8%)	13 (36.1%)
	Muscle/eyelid/conj	3 (15.8%)	1 (5.9%)	4 (11.1%)

2.5.3 **Patient characteristics**

There were 15 (41.7%) males and 21 (58.3%) females in the GPA group (Figure 2.3) with an average age of 50.35 SE 2.87 (i.e. 14-87 years old). Of the 36 patients managed as orbital GPA, 19 (52.8%) had a previous diagnosis of GPA (known GPA) from other organ manifestations of the disease and had received or were still on treatment (prednisolone with or without second line treatment). (Table 2.3) Ocular presentation was the initial symptom of GPA in the remaining 17 (47.2%) patients. (Figure 2.4)

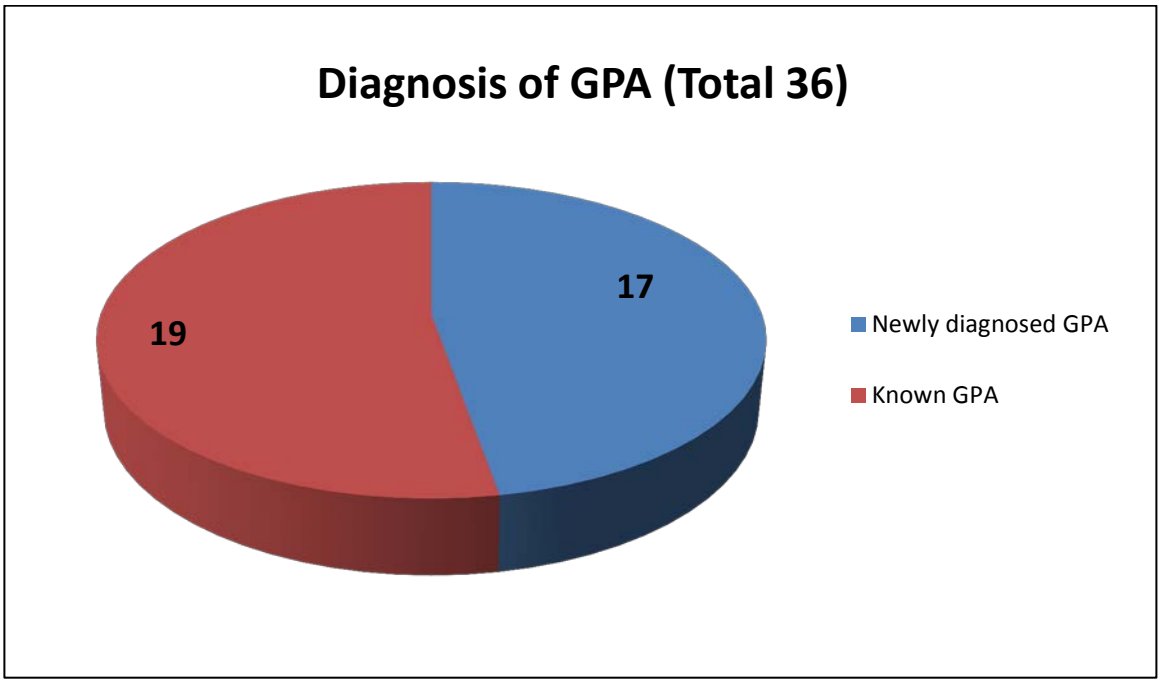


Figure 2.4: Distribution of patients according to newly diagnosed or known (recurrent) GPA.

2.5.4 GPA Classification and Organ Involvement

Based on our definition, 34 patients fulfilled our criteria for localised GPA and two patients were classified to have systemic GPA as they had been previously diagnosed as renal GPA (Figure 2.5)(Table 2.3). The majority of cases (n=30, 83.3%) had some degree of sinonasal involvement together with their orbital disease. Only 6 (16%) out of the 36 patients had the disease confined to the orbit only. (Table 2.2)

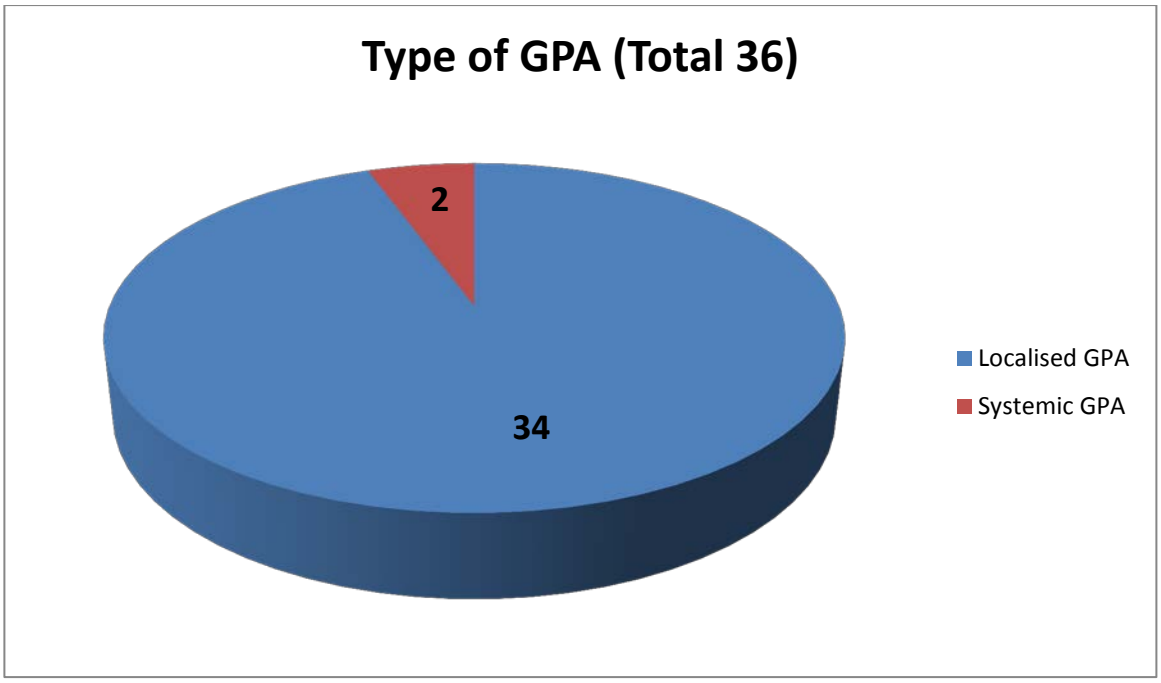


Figure 2.5: Distribution of patients according to type (form) of GPA.

2.5.5 **Ocular Presentation**

Ocular presentation was observed to be more severe in the newly diagnosed GPA group. The most common ocular presentation in orbital GPA patients were proptosis (n=17, 47.2%), lid swelling (n=14, 38.9%), diplopia (n=14, 38.9%) and reduced ocular motility (n=12, 33.3%). These were seen particularly in the newly diagnosed GPA patients (n=10, 58.8%).Symptoms from the nasolacrimal duct system also predominates (n=16; 44.4%) but in contrast, it is mainly contributed from known GPA patients (n=14, 73.7%). In up to half the patients (n=9, 52%) in the newly diagnosed group presented with concurrent reduced vision where in the known GPA group, none of these patients complained of reduced vision on presentation.(Figure 2.6)

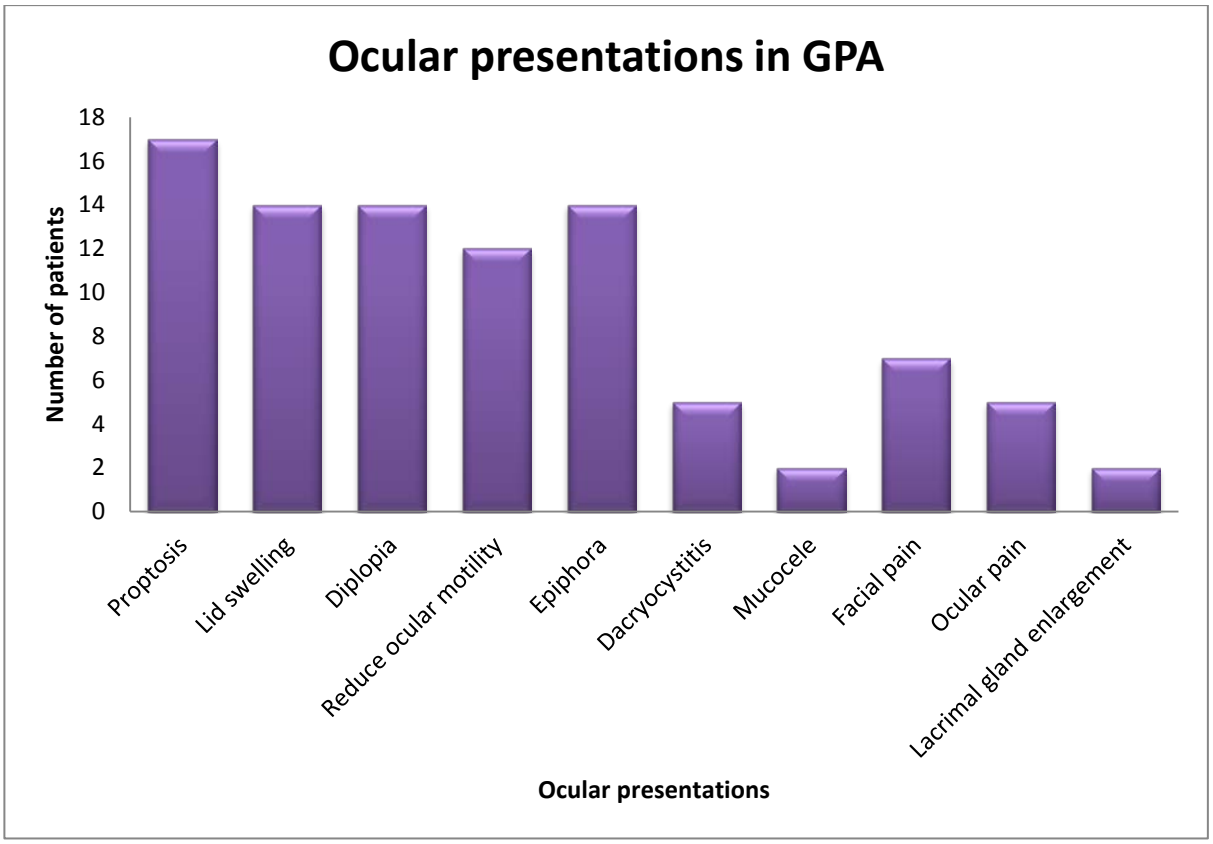


Figure 2.6: Ocular presentations in GPA group.

In known GPA patients, epiphora secondary to nasolacrimal duct obstruction was the main clinical presentation, occurring in 14 (73.7%) of the 19 patients. Five of these patients also presented with dacryocystitis and two with mucocele. Interestingly, facial symptoms such as facial pain or facial numbness only occurred among patients in the known GPA (n=7, 37%) and not in newly diagnosed GPA patients. Proptosis, diplopia and lid swelling occurred in seven patients in the known GPA group (six localised GPA and one generalised GPA). Five of these patients had associated ocular pain and two had lacrimal gland enlargement. (Figure 2.6)

Lacrimal gland involvement occurred in nearly a third of the patients (n= 11, 30.5%). In two out of the 11 patients, lacrimal gland enlargement was the initial manifestation of orbital GPA. Epiphora due to nasolacrimal duct inflammation and block was the initial

presentation of GPA in one patient, with no other signs of orbital or sinus involvement. In this patient, the diagnosis of GPA was based on the CT appearance of the paranasal sinuses where extensive bony destruction was seen despite not previously having any nasal surgery. In addition, ANCA titres were also found to be elevated.

2.5.6 **Orbital Structure Involved**

In most cases tissues for biopsies were taken from a combination of orbital structures. However, biopsies were mainly obtained from orbital masses (n=21, 58.3%) and the nasolacrimal duct (n=13, 36.1%). Eleven (30.5%) out of the 36 GPA patients had lacrimal gland enlargement clinically where biopsy tissue from this structure was taken. (Table 2.2)

2.5.7 **ANCA status**

Only 21 (58.3%) patients were documented to be ANCA positive at some point of the course of their disease, where GPA was localised in the orbit and sinonasal in 10 (27.8%) patients. Only 13 (36.1%) of the 36 patients investigated during their first ocular presentation were ANCA positive; seven from patients with pre-existing GPA and six from the newly diagnosed group. Five patients with pre-existing GPA who were previously ANCA positive were not ANCA positive during their orbital presentation, and three patients in the newly diagnosed GPA further became ANCA positive during the course of their disease.

2.5.8 **Treatment and Follow Up**

In addition to ophthalmology review, patients were also evaluated and managed, where necessary, by physicians with special interest in vasculitis and GPA.

All patients received corticosteroid as the mainstay of treatment. Interestingly, seven patients (all in the newly diagnosed group) were successfully stabilised with only systemic corticosteroids where six were successfully tapered off treatment. The remaining one patient was maintained on low dose oral corticosteroid.

The majority of patients had second line immunosuppressants which included azathioprine, methotrexate or mycophenolate mofetil for remission induction and remission maintenance therapy. Tacrolimus was used for remission maintenance in one patient. A total of 14 patients received cyclophosphamide during the course of their disease and four of these patients received an additional treatment with Rituximab due to disease recurrence. Eight patients in total had received Rituximab where RTX was the initial treatment of choice in four patients, instead of cyclophosphamide, to achieve disease remission. Only one patient was given an additional treatment of cyclophosphamide after a previous course of Rituximab due to disease recurrence. A total of seven patients underwent orbital mass debulking.

14 patients had dacryocystorhinostomy (DCR) for nasolacrimal duct block and all patients received post-operative steroid treatment. Repeat DCRs were performed in three patients where additional second line immunosuppressant was given during the subsequent procedures to better control the post-operative inflammation. In the majority of these patients, additional or increasing second-line immunosuppressive treatment was not necessary.

2.5.9 **Outcome**

The main aim for this section of the study was to identify patients with orbital GPA who progressed into the systemic form of the disease i.e. from orbital GPA progressing to involve major organs and becoming life threatening, such as the kidneys, overtime. In this study however, retrospective case note reviews of all the 36 patients with orbital GPA,together with information gathered from general practitioners and other medical specialities from other hospitals, revealed that to date, patients who presented with localised orbital GPA with no pre-existing systemic involvement, did not progress to the systemic form overtime. Therefore, in this sample group, in patients with localised GPA, the disease remained localised within the orbit and did not spread to involve other organs. In contrast, patients with systemic disease however, may further develop orbital manifestations as seen in our 2 patients in the known GPA group.

At the two year follow up mark, we found that nine out of the 36 patients (25%) had further deterioration of vision throughout their follow up; six were due to cataract formation, two due to recurrent orbital inflammation and one patient developed a refractory painful red blind eye, which was eventually enucleated. The three patients with worsening vision due to recurrence and refractory blind eye were patients in the known GPA group. All other patients had their pre-inflammation vision restored. 30 patients remained on maintenance immunosuppression. In six patients, it was possible to halt treatment. All patients who underwent DCR had good post-operative outcome with resolution of their problem although three patients had to undergo repeat procedures.

2.6 Discussion

Our hospital is a tertiary referral centre where we receive referrals for orbital inflammation disorders and ocular manifestation of GPA. Our retrospective study goes back to a period of 21 years where we reviewed 36 patients who were diagnosed and managed as orbital GPA with a minimum follow up of two years from their orbital presentation. Our aim was to look at their clinical presentations, course of management and clinical outcomes. In particular, the main aim for this section of the study is to identify patients with orbital GPA who progressed to the life threatening systemic form over time, and compare their clinical presentations with those who remained with localised disease.

2.6.1 Clinical presentation

It is known that orbital GPA occurs either;(1) solitary as ocular vasculitis or orbital mass (granuloma) formation or (2) as disease extension from adjacent affected structures, such as paranasal sinuses (contiguous spread), or (3) as part of an overall manifestation of the systemic disease (Pakrou et al., 2006). In this study, our group of patients could also be divided into two main groups; (1) patients newly diagnosed with localised GPA from their orbital presentation i.e. *de novo,* and (2) patients with GPA presenting with an orbital extension/manifestation of the disease i.e. from disease spread from adjacent structures or systemic form of the disease.

Orbital and adnexal manifestations of GPA are variable, and typical presentations include orbital mass or proptosis (69%), nasolacrimal duct obstruction (52%), limited ocular rotations (52%), lid erythema and oedema (31%), bony destruction (21%), and reduced visual acuity (17%) (Woo et al., 2001)(Tarabishy et al., 2010). In our group of patients, the orbital clinical symptoms and signs are more severe if the orbit was the initial

manifestation of GPA (newly diagnosed GPA) compared to recurrent cases or disease spread from other structures (known GPA). These include proptosis, pain, globe displacement, limitations in ocular motility and reduced vision. Proptosis due to a retrobulbar orbital mass was the most common clinical finding in newly diagnosed GPA patients. Despite this, surprisingly, there were no permanent visual impairments in the majority of these patients.

Nearly a third of the patients (i.e. 11 out of 36) in our study group presented with lacrimal gland involvement as an initial manifestation of orbital GPA. Although there have been few reports of similar findings in GPA patients in the past, the number of cases were generally small (Soheilian et al., 2002)(Perry et al., 1997)(Leavitt and Butrus, 1991). It is interesting that in one patient, recurrent nasolacrimal duct block with no other orbital, ocular or sinus involvement was an initial presentation of GPA. It has been reported that NLD obstruction is not always due to contiguous spread from the sinuses, but it may be a direct effect of focal GPA vasculitis in the lacrimal drainage system (Ghanem et al., 2004).

Patients with a previous history of GPA (known GPA) in our study group, generally had milder orbital symptoms, mainly affecting the nasolacrimal drainage system; NLD block. These patients were usually referred for the management of epiphora where five patients had associated dacryocystitis and two with mucocele. Contiguous spread from affected sinuses can cause nasolacrimal duct inflammation and blockage leading to symptoms of epiphora and dacryocystitis. This problem is reported to be typically a late manifestation of GPA and affects approximately 7% of patients (Eloy et al., 2009). Dacryocystorhinostomy (DCR) has been shown to be a safe and successful procedure for this problem (Kwan and Rose, 2000). However, DCR is not recommended in GPA if the disease is still active due to the risk of recurrence and disease extension to the orbit and skin (Eloy et al., 2009). All our patients were successfully treated with DCR and in nearly all patients, additional or increasing second-line immunosuppressive treatment was not necessary. This may be due

to the fact that these patients had previously received treatment for the GPA and thus disease manifestations were milder.

However, it is important to point out that some patients with pre-existing GPA (known GPA) with localised GPA and was already on treatment, presented severe orbital symptoms such as proptosis, diplopia and ocular pain. This suggests that localised disease manifestation can still extend to involve adjacent structures despite being on treatment, hence indicating local inflammatory activity is still uncontrolled and on-going. Furthermore, it has been reported that in patients with known GPA, signs of ocular inflammation may indicate an active disease in other organs although there were no systemic symptoms observed. Therefore, although the ocular symptoms may be mild, further investigation to determine the extent of the disease systemically is important (Tarabishy et al., 2010).

2.6.2 **ANCA status**

GPA has been established to have a strong association with c-ANCA (Rao et al., 1995). In our sample population, more than half of the patients (n=21, 58.3%) were ANCA positive at presentation where nine were from the newly diagnosed group and 12 from the known GPA group. This is aligned with other earlier reports where it is stated that in limited GPA about 50-65% of patients will demonstrate ANCA positivity (Tarabishy et al., 2010)(Lamprecht and Gross, 2007). However, only a third of our patients were found to be ANCA positive at the time of presentation.Patients who are ANCA negative at presentation can become positive over time. Conversely, patients with GPA who were ANCA positive in previous serology tests may show a negative ANCA result on repeat testing, despite clinically presenting an active orbital GPA. This demonstrates that although ANCA titers are useful in the diagnosis of GPA, in localised GPA they can be unpredictable and

therefore unreliable for diagnosis and disease management. Alternatively, this also suggests that serology testing for ANCA should be done periodically in patients with orbital inflammatory diseases particularly those with recurrent attacks as it may manifest later in the disease course.

2.6.3 **Treatment regimes**

In most patients, second line immunosuppressant was given and showed good control of the disease. Interestingly, seven patients were successfully stabilised with only corticosteroid. In 2010 Watkins et al, reported that the majority of their patients with ANCA positive ocular diseases were treated with systemic corticosteroids, and over three-quarters of the group were prescribed systemic immunosuppressive medications, indicating a quarter of the patients did not receive other immunosuppressive treatment other than corticosteroid (Watkins et al.). In another study however, only one out of 10 patients with orbital GPA achieved good control with remission, with corticosteroid therapy alone and the majority had to receive a combination of a systemic immunosuppressive medication and corticosteroid (Perry et al., 1997). These findings suggest that the treatment for orbital GPA varies between individuals and needs to be judged and adjusted based on the clinical presentations.

2.6.4 **Long term outcome**

Nine of the 36 patients developed deterioration in vision due to various complications secondary to GPA. Although poor vision was not the main presenting symptom in the known GPA group, patients who developed poor vision overtime were mainly from this group, particularly in those with recurrent orbital inflammation. Orbital mass in GPA in

particular, have been described before as a refractory form of GPA which can cause severe visual loss (Holle et al., 2013b). Therefore in orbital GPA, it appears that prompt diagnosis and treatment is crucial during the initial presentation phase or within the earlier stage of the disease, as even though symptoms may be severe but early aggressive treatment can be organ and sight saving in the long run. Episodes of recurrent orbital inflammation that could occur due to diagnostic delay and inadequate treatment may result in permanent organ damage and visual loss overtime. This further highlights the need for early disease detection and intervention with the appropriate treatment.

More importantly we discovered that in our two year follow up; all patients with localised GPA did not show progression to the generalised or severe form. This echoed 2 previous published studies by Fechner, 2002 and Holle, 2010. Fechner et al reported that their patients with only orbital involvement of GPA did not develop systemic progression of Wegener's granulomatosis throughout their follow up (Fechner et al., 2002). However this study was fairly small compared to our study, where only 15 GPA patients were involved; 12 with orbital GPA. Similarly, Holle et al in 2010, in their large study, found that none of their patients with localised GPA progressed to the severe generalised disease stage (creatinine > 500 micromole/l)(Holle et al., 2010a). Nonetheless, it should be noted that unlike our study which is only limited to the orbit, in their study, the criteria for localised GPA were a combination of the head and neck, orbit and the respiratory tract.

In general, it is therefore possible that orbital GPA does remain localised and does not progress to systemic GPA over time. It also appears that patients with systemic GPA had the systemic manifestations of the disease prior to orbital symptoms. The lack of progression of the disease from the orbit to other organs could be a result of prompt disease recognition and management, or newer and better treatment regimens.

2.7 Conclusion and Further Plan

Our initial findings show that the presentations of orbital GPA vary. The course of management for these patients generally requires clinical judgement based on individual presentations and is catered according to individual requirements.

Our main finding in our patient population was that no patients showed progression of the disease. Localised form of GPA remained localised and do not progress to systemic GPA. On the other hand, we also found that extension of pre-existing GPA from other organs to the orbit is not uncommon despite patient receiving treatment. We also discovered that newly diagnosed orbital GPAs have more severe ocular presentations compared to known GPA but yet have good visual outcomes, suggesting that early and appropriate treatment results in positive clinical results. In contrast, patients with poor visual outcomes were within the known GPA group i.e. patients with recurrent GPA, who have received or are on treatment.

It is difficult to conclude whether early intervention or treatment prevents disease progression in cases of localised GPA or whether localised manifestation of GPA is actually a separate entity from the generalised systemic form. Nevertheless, early diagnosis, prompt management and intervention during the orbital stage are still important as it appears that the long-term outcomes in most of these patients are generally encouraging. This will also reduce the need for long-term maintenance therapy. As a result, this would lessen patients' exposure to cytotoxic drugs and therefore reduces side effects. In addition, it would be more cost effective if long-term maintenance therapy can be reduced.

Our primary aim in this study was to look for any cell or tissue changes that could act as a biomarker for disease progression in orbital GPA. However, since the disease remained

localised in all of our patients, it was therefore no longer possible to investigate and determine these histological features. Nevertheless, our initial findings did raise further questions with regards to orbital GPA, which include: (1) is orbital GPA a true distinct disease entity or could it be a severe form of idiopathic orbital inflammatory disease? (2) Are there actual cellular and histological differences between orbital GPA and other OIDs? (3) If there are, what particular features may be different and could they be used clinically as a disease biomarker for the diagnosis of orbital GPA, thus helping in early diagnosis? (4) Are there any histological difference between known GPA and newly diagnosed GPA?

We therefore proceeded to explore these questions by comparing the histology of orbital GPA with other OIDs.

3 Chapter 3: Histology of Ocular GPA Compared to other OIDs

3.1 Overview and Objective

Histology investigations regularly play an important role in clinical practice. Histology analysis from tissue biopsy could help in establishing the diagnosis, confirming a clinical suspicion, detecting disease progression and in monitoring treatment response. Therefore, understanding the histological features and recognising the pattern of tissue responses that characterises a particular disease is vital. In the management of OIDs, tissue biopsy is always performed whenever possible.

It is first useful to understand the cells involved during an inflammatory process, which is the focal feature in these orbital diseases. Generally, inflammation is a body response by vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. The inflammatory response is a multifaceted biological process involving interactions between a vast amount of various cellular, protein and biochemical substances. The human inflammatory process can be broadly divided into two; acute inflammation and chronic inflammation. GPA, IOID and sarcoidosis are chronic inflammatory diseases.

3.2 The Acute Inflammatory Process

The acute inflammation is an immediate response to injury or attack by invading micro-organisms. It constitutes several components that include tissue damage, vascular changes, activation and adhesion of leucocytes to the vascular endothelial wall, and leucocyte emigration to the site of injury.

The initial immune cells involved in the early stage of this process are cells from the innate immune systems, namely, the local macrophages, histiocytes, dendritic cells and Kupffer cells. These cells possess certain receptors that recognise foreign antigens and become

activated upon invasion or tissue damage. Together with the damaged tissue and also bacterial products, these cells release various inflammatory mediators such as bradykinin, histamine, free radicals, prostaglandins, etc. This then results in local tissue changes which include vascular dilatation leading to increased blood flow resulting in redness (rubor) and increased local heat (calor), increased vascular permeability causing extravasation of plasma fluid along with inflammatory cells leading to tissue swelling (tumor). There is increased sensitivity to pain (dolor) and local tissue functional loss may occur (funtio). These features represent the classical features of acute inflammation and are usually the clinical presentation manifested by patients.

Neutrophils are the main cellular components during acute inflammation. During active inflammation, neutrophils in the blood vessels adhere to the vascular endothelium wall and migrate through the permeable vascular wall towards the site of injury. Together with macrophages, neutrophils neutralise the offending body and remove injured tissue. Apart from neutrophils, other effector cells involved in the acute inflammatory phase include lymphocytes, mast cells and basophils.

In addition to cellular activity, the inflammatory process is also aided by a series of biochemical cascades by non-cellular proteins in the plasma such as the complement system and fibrinolysis systems, to initiate and propagate the response. The acute inflammatory response ceases once the stimulus has been removed as it requires constant stimulation to be sustained.

3.3 The Chronic Inflammatory Process

The term “chronic inflammation” is used when the acute inflammatory process does not cease and the inflammatory process persists. The prolonged inflammatory process leads

to a progressive shift in the type of cells present at the site of inflammation, and simultaneous destruction and healing of the tissue from the inflammatory process is characteristic of chronic inflammation.

The main effector cells in chronic inflammation are macrophages. When a pathogen is not successfully removed, macrophages together with lymphocytes and other inflammatory cells surround the organism. These inflammatory cells form a wall around the pathogen to limit its activity or damage, creating a ball-like structure known as “granuloma”. Macrophages then remove the engulfed pathogen via digestion. In other instances, macrophages tend to group together to form one large cell known as giant cells. Macrophages tend to also alter their shape during an inflammatory process, and could resemble other cells such as the epithelial cells termed epithelioid cells, and Langerhan cells. The formations of granuloma are also seen in other auto-immune diseases which are not related to pathogen invasions such as sarcoidosis. Usually, where inflammation is related to macrophage dominance via giant cells or granuloma formation, this is termed as “granulomatous inflammation”. GPA is described as a granulomatous inflammatory disease.

3.4 The Various Morphologic Patterns in Inflammation

3.4.1 Granulomatous inflammation

Granulomatous inflammation is a distinct type of chronic inflammation where activity of cells from the mononuclear phagocyte cell lineage predominates. The inflammation is characterised by collection of macrophages, multinucleated giant cells and epithelioid cells. Granulomas, as described above, are present. Nevertheless, more diffused pattern

of cell aggregation is not uncommonly seen in granulomatous inflammation. Granulomata can frequently have a necrotic centre surrounded by macrophages with a cuff of lymphocytes surrounding it. This Inflammatory reaction is influenced by cytokines such IFN gamma, lymphotoxins, IL-3, GM-CSF and IL-12. In response toIL-12 drive, the immune response shifts towards T- helper 1 T cell (Th1 T cells) mediated immune response rather than T-helper 2 (Th2 T cells) reaction. TNF also play a role in granuloma formation, where in the absence of TNF secreting macrophages, granulomas are seen not to form. Granulomatous inflammation is often seen as a Type 4 Hypersensitivity reaction and manifests in a variety of conditions such as allergy, infection with tuberculosis, leprosy and syphilis, and autoimmune disorders such as GPA and sarcoidosis. Granulomatous inflammation is also known to occur in auto-immune and neoplastic diseases as well as conditions of unknown causes (William 1983).

3.4.2 **Fibrinous inflammation**

Fibrinous inflammation can be an acute inflammation but most often is a chronic response. It is characterised as inflammation with fibrin deposits. This often results from severe injury to blood vessels where significant increase in vascular permeability allows fibrin to pass through the blood vessels. This type of inflammation is commonly seen serous cavities such as in the pericardial cavity, causing restrictive fibrosis, pleural space and peritoneal cavity resulting in adhesions.

3.4.3 **Purulent inflammation**

Inflammation associated with pus formation is known as purulent inflammation. At the site of inflammation, collection of large amounts of neutrophils with necrotic tissues and fluid

are seen. Abscesses are described as pus enclosed by surrounding tissues. Purulent inflammations are usually associated with pyogenic bacterial infections such as staphylococci. It has been described that in GPA, foci of microabscesses, where a collection of neutrophils are seen within necrotic tissues, together with surrounding granulomatous inflammation.

3.4.4 Serous inflammation

Serous inflammation is characterised by the large outpouring of non-viscous serous fluid derived from blood plasma. The fluids are commonly secretions from mesothelial cells of peritoneal, pleural or pericardial linings. Viral infection resulting in skin blisters are an example of this inflammation.

3.4.5 Ulcerative inflammation

Ulcerative inflammation occurs mainly near an epithelial surface. The inflammatory process can lead to tissue necrosis causing exposure of lower layers. Ulcers are epithelium excavation resulting from the inflammation.

3.5 Potential Outcomes of Inflammation

The end result or sequalae from an inflammatory reaction relies largely on the tissue type that is injured as well as the mechanism or agent that incite the inflammatory process. In general, the outcome of inflammation can either be complete resolution and restoration of

normal tissue function, fibrosis, abscess formation or persistent chronic inflammation. These changes can be seen to occur even during an ongoing active inflammatory state, particularly in severe and/or chronic inflammation.

3.5.1 **Resolution**

Complete resolution usually occurs in mild and short lived inflammatory conditions. Resolution is said to occur when the inflamed tissue is restored to its normal status and changes that are associated with inflammation such as vasodilatation and cellular and biochemical infiltration, cease. Tissue damage is removed and cells regenerate, restoring normal structure as well as tissue function.

3.5.2 **Fibrosis**

Fibrosis usually occurs when there is large amount of tissue damage. In this situation, tissues generally are unable to regenerate leading to fibrous scarring made up of collagen. Scarring is devoid of specialised structures thus tissue function at that site is lost.

3.5.3 **Abscess Formation**

Pus collection in a cavity or tissue is called an abscess. It is usually associated with infection. The pus is seen to be made of dead neutrophils together with bacteria and debris of destroyed tissues.

3.5.4 Chronic inflammation

As previously mentioned, chronic inflammation occurs when the inciting agent causing the inflammation persists and is not removed during the acute inflammatory phase. This process could continue over days, months and even years leading to continuous local tissue damage. Macrophages are the predominant cells seen in chronic inflammation. These cells have the ability to produce toxins, used to defend against the pathogen. However, these toxins may also cause normal surrounding tissue damage and as a consequence localised tissue destruction such as necrosis can occur

3.6 Inflammatory Cells and Tissue Changes seen in GPA Biopsies

The histopathology of GPA was first described by Frederick Wegener in the 1930s based on post mortem lung examination of patients with systemic GPA. The histology of GPA was seen to consist of a variable mixture of granulomatous lesions with microabcesses or fibrinoid necrosis, tissue necrosis and vasculitis of small to medium sized vessels. Interestingly, it is reported that granuloma formations are seen to occur early in the disease process with vasculitis developing in the later stages (Wegener 1990).

3.6.1 Lung histology in GPA

In the lungs, the histology of GPA is reported to be divided into major and minor pathologic manifestations. Major manifestations are described to be the classical appearance of GPA histology and were of use diagnostically. In contrast, minor manifestations were histologic

changes observed in association with classical GPA lesions, but were inconspicuous and deemed unuseful for diagnostic purposes.

The major pathologic features in GPA include (1) parenchymal necrosis, (2) vasculitis, and (3) granulomatous inflammation accompanied by an inflammatory infiltrate composed of a mixture of neutrophils, lymphocytes, plasma cells, histiocytes, and eosinophils. The majority of patients (85%) were described to have parenchymal necrosis either seen as microabscesses or as geographical necrosis. The necrotizing granulomatous lesions consisted of poorly formed granuloma and scattered giant cells.

Minor pathologic changes included interstitial fibrosis, alveolar hemorrhage, tissue eosinophils, organizing intraluminal fibrosis, endogenous lipoid pneumonia, lymphoid aggregates, and a variety of bronchial/bronchiolar lesions (including acute and chronic bronchiolitis), follicular bronchiolitis, and bronchiolitis obliterans. Interestingly, eosinophils were found to be present in 100% of these cases although classified as a minor manifestation (Travis 1991).

3.6.2 **Renal histology in GPA**

Kidneys affected by GPA are typically described as segmental pauci-immune crescentic necrotizing glomerulonephritis. In the early stages of the disease, thrombotic changes in the glomerular capillary loops may be seen. Thereafter, segmental fibrinoid necrosis with nuclear debris and disruption of the glomerular basement membrane occurs. In due course, cellular crescent is seen formed, collapsing the remaining glomerular tuft and tissue necrosis ensue. Granulomatous inflammation is generally not typical in renal GPA and cellular infiltrates seen are predominantly mononuclear leucocytes with moderate

infiltration of neutrophils. The presence of vasculitis however, is not typically observed in renal biopsy specimens affected by GPA.

3.6.3 **Ocular histology in GPA**

In the limited form of GPA like in the orbit and nasal cavity, classical histological features of GPA are not always apparent in the majority of cases and have been reported to only occur in 16-25% of patients with upper respiratory GPA (Raynaud et al., 2005) and 54% in orbital GPA (Kalina et al., 1992). Despite this, clinically, orbital GPA could still present severe ocular symptoms and signs such as pain and proptosis that could result in permanent ocular morbidity like diplopia, reduced vision and even blindness. Indeed, orbital GPA has been described to represent a refractory form of GPA which could result in grave ocular consequences (Holle et al., 2013b).

The pathologic features of biopsies from patients with ophthalmic GPA have been previously described. The histology of ophthalmic GPA is associated with granulomatous foci, collagen necrosis, neutrophil/nuclear dust, plasma cells and infiltrating eosinophils (Ahmed et al., 2008). The presence of granular degeneration of the interstitial collagen, mummification of the collagen with the disappearance of fibroblastic nuclei, and a polymorphous infiltrate exhibiting plasma cells, lymphocytes, neutrophils and eosinophils within the epithelioid granulomas, are thought to be particularly indicative of the diagnosis of GPA (Ahmed et al., 2008).

3.7 Ocular Histology in Sarcoidosis

The histology of sarcoidosis typically shows a non caseating granulomatous inflammation with presence of foreign giant cells. Infiltrating immune cells in tissue biopsies are similar to that of GPA but in sarcoidosis, well formed granulomatous nodules (granuloma) are typically seen and are approximately the same size. Features of necrosis can also be observed. The presence of asteroid bodies which are star shaped acidophilic bodies within the tissues can be seen although their presence is not pathognomonic with the diagnosis of sarcoidosis.

3.8 Ocular Histology in IIOD

The histology of IIOD is non-specific and diverse (Yuen and Rubin, 2003). Tissues are seen to have a varied polymorphous immune cellular infiltration (neutrophils, plasma cells and macrophages). It can also portray a typical granulomatous inflammation like pattern, display tissue eosinophils, as well as infiltrative sclerosis (Fujii et al., 1985).

3.9 Ocular Histology in TED

Orbital tissues in TED are characterised to portray lymphocytic infiltration, focal aggregation of B cells, plasma cells and mast cells. In the orbit, accumulation of glycosaminoglycan can also be observed as well as an increased in adipocyte differentiation.

3.10 Objective

The histological features of ocular GPA previously described are not truly pathognomonic for orbital GPA as other orbital inflammatory diseases, such as sarcoidosis and idiopathic inflammatory orbital diseases, may also display similar features. We therefore wanted to investigate the histological features that are associated with the clinical diagnosis of orbital GPA; using both histopathology reports and the tissue biopsies, and compare them to the histology of other OIDs. In addition, we wanted to also identify any features in the histology of orbital GPA that may not be present in the biopsies of OIDs or those which may have greater presence in orbital GPA biopsies.

The main aims in this part of the study were to:

1) Examine subjectively if there is a difference in the cellular occurrences and tissue changes between GPA and non-GPA orbital biopsies based on histopathology reports.

2) Objectively identify and quantify the different cell types and tissue changes present in orbital biopsies of patients diagnosed with orbital inflammatory disease.

3) Compare the amount (by count) of the different cell types and tissue changes, between patients diagnosed with GPA with OIDs.

4) Compare the amount (by count) of the different cell types and tissue changes, within the GPA group.

3.11 Method

3.11.1 Patient selection

All previously identified patients were included in the study. Patients were allocated the same study numbers to maintain anonymity and we followed the same definition of terms as before.

3.11.2 Subjective Comparisons with Histopathology Reports

Histopathology reports were obtained from the medical case notes and each report were allocated a study number corresponding to the patients study number. The diagnosis for each report was also masked. The biopsy reports were then reviewed. The cell types and tissue responses were documented qualitatively as present (+ve) when described in the report, or absent (-ve) when not described in the report. Cell types that were noted in the histopathology reports included neutrophils, eosinophils, lymphocytes, macrophages, plasma cells, giant cells and mast cells. The tissue responses noted include nuclear debris, granulomas, vasculitis, necrosis and fibrosis. (Appendix A)

3.11.3 **Objective Comparisons with Orbital Tissue Biopsies**

3.11.3.1 Tissue Selection

Tissue biopsies, in paraffin block preparation from patients included in the study, were obtained from the Pathology Department, Institute of Ophthalmology, University College of London (UCL) archive storage. The blocks were anonymised by using the pathology numbers which matched an allocated patients' study numbers. This also masked the diagnosis for each block.

3.11.3.2 Slide Preparations and Haematoxylin and Eosin (H&E) Stainings

Haematoxylin and eosin (H&E) staining was performed on all blocks for general cellular and tissue morphological definition and analysis. A sledge microtome was used for tissue sectioning and blocks were cut at a thickness of 5 micrometers and then placed on Superfrost plus object glass slides. Slides were only labelled by the patients' pathology numbers to maintain anonymity and masked the pathology. The object slides were then left on a hot plate for 60 minutes at 40°C to encourage good tissue adherence to the slide and were then further incubated overnight at 37°C before use. H&E staining was performed for all slides on the same day and under the same laboratory conditions as per laboratory protocol via the automated H&E staining machine. Slides were then mounted with DPX mounting medium and cover slips and allowed to dry overnight.

3.11.3.3 Imaging and Objective Cell and Tissue Counts

The images of the whole slide from every biopsy were taken with the Hamamatsu Nanozoomer Digital Pathology Scanner and captured images were saved using pathology numbers to maintain patients' anonymity and the diagnosis masked. Cell and tissue counts were done masked to the patient's identity as well as disease diagnosis. Inflammatory cells and tissue changes observed on the slide images were then counted using the ImageScope image analyser and ADCIS® Stereology Toolkit. Cell count was performed for lymphocytes, plasma cells, eosinophils, neutrophils, mast cells, macrophages and giant cells. Tissue changes were characterised as nuclear debris, granulomas, vasculitis, fibrosis and necrosis. (Appendix A)

Cell counting was performed using the ADCIS® Stereology Toolkit frame count programme. The region of interest was first identified manually from the image of the tissue section. This was done by drawing around the border of the whole tissue section. The software was then programmed to generate 20 random counting frames within the region of interest. Figure 3.1 Each counting frame measured 1mm x 1mm and consisted of two inclusion lines (right and top borders) and two exclusion lines (left and bottom borders) resulting in a total area of 20mm^2 analysed for every slide. This was then repeated in all slide images to ensure that the total area analysed were standardised on all images. Cells lying within the frame or on the inclusion line and not touching the exclusion line were included in the count. Each different cell types identified were appointed different colour codings and marked by hand (e.g. red for neutrophils, green for lymphocytes, etc.)(Figure 3.2). This was performed in all 20 frames. At the end of the 20 frames, the software then generated the total number of cells marked for each different cell type to produce the total count of each cell type in that slide image.

For quantifying tissue changes, the ADCIS® Stereology Toolkit point count programme was also used. As previously described, the region of interest was identified from the slide image manually. 20 random points were programmed to be generated by the software within the region of interest. Figure 3.3 Different tissue changes were assigned to different colour codings. The points were then marked with the different colour code if it 'hits' (or marks) on a particular tissue change. This was performed on all 20 points. The computer then generated the number of 'hits' for the different tissue changes. Figure 3.4

3.11.4 **Image analysis**

Figure 3.1: Image analysis for objective cell count using a H&E GPA tissue at 20x magnification. A total of 20 random counting frames (blue frames), each 1mmx1mm (1mm^2), generated by the ADCIS® Stereology Toolkit within the region of interest (marked red).

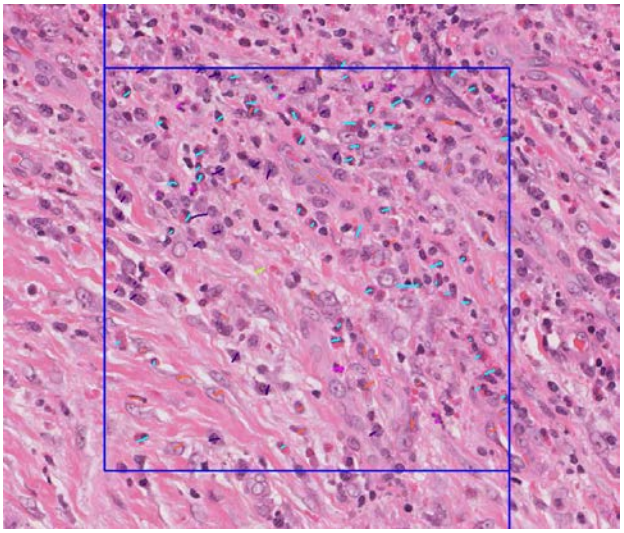


Figure 3.2: Example of frame counting for cell count on a H&E GPA tissue at 400x magnification. Each counting frame measures 1mm x 1mm and consists of two inclusion lines (right and top borders) and two exclusion lines (left and bottom borders). Each different cell types are identified by hand and marked using different colour codings. Only cells within the frame and not touching the exclusion lines were included in the count

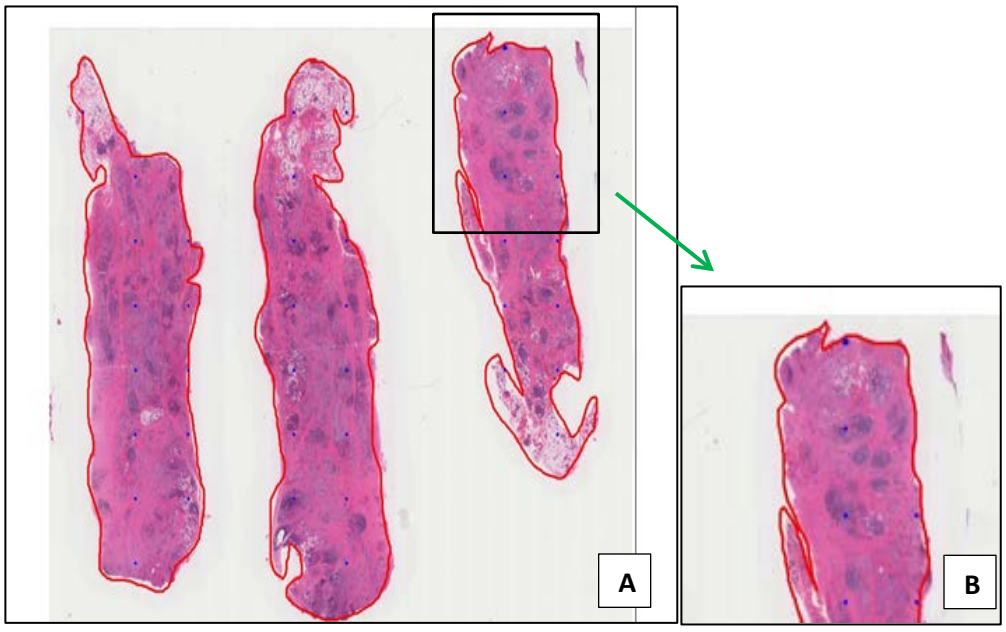


Figure 3.3: Example of tissue count with point counting on a H&E GPA tissue A: Image analysis for tissue changes at 20x magnification. A total of 20 random point counts (blue x) within the region of interest (marked in red) B: Enlarged section (40x magnification) demonstrating generated "blue x".

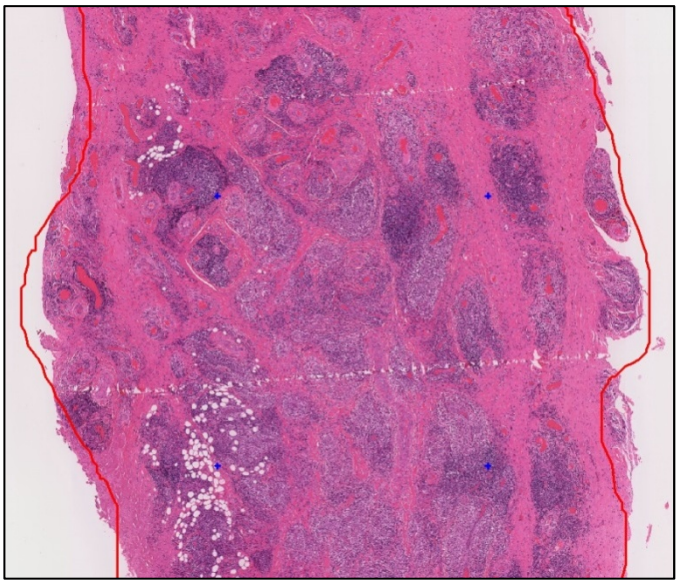


Figure 3.4: Point counting for tissue changes (image at 100x magnification). Different color codings are assigned to different tissue changes. The points are then marked with a different colour code if it 'hits' (or marks) on a particular tissue change. Above is an example of a 'zero score', where none of the points are marking a tissue change.

3.11.5 Validation process for cell and tissue counts

Identification of cells and tissue changes were counter checked and validated by a senior pathologist (secondary supervisor) who was also masked from patients' identities and diagnoses. A portion of the slides were also re-counted to ensure total counts were reproducible.

3.12 Data Analysis

3.12.1 Subjective Histopathology Analysis

Comparison of the total number of reports with positive presence of the cell types and tissue changes were then compared between the GPA and non GPA i.e. other orbital

inflammatory diseases (OID), via the Mann Whitney test with SPSS17. Further sub-analysis within the GPA group was also conducted with the Mann Whitney test SPSS17.

3.12.2 **Objective Tissue Biopsy Analysis**

Cell counts were entered into an excel data sheet where anonymised pathology numbers were then matched to the corresponding study number for each patient. Patients were then divided into two groups – clinically diagnosed orbital GPA and OIDs and cell and tissue counts were compared.

Further sub-analysis within the orbital GPA group was also performed.

3.12.3 **Statistical Analysis**

Student's t-test and Fisher's exact test were performed with GraphPad Prism 5 and SPSS17 for comparison between groups during the subjective analysis with histopathology reports. The Mann Whitney test was performed with GraphPad Prism 5 for comparison of the cell and tissue count on tissue biopsies between the groups. Multivariate analysis was performed with SPSS17 to look for any independent association of a cellular profile or tissue response with the diagnosis of GPA for both subjective and objective analysis.

3.13 Results

3.14 Histopathology Report (subjective analysis)

3.14.1 GPA versus OID

234 histopathology reports from all patients previously identified and included in the study were reviewed. In general, the histopathology reports showed a mixed pattern of acute and chronic inflammation.

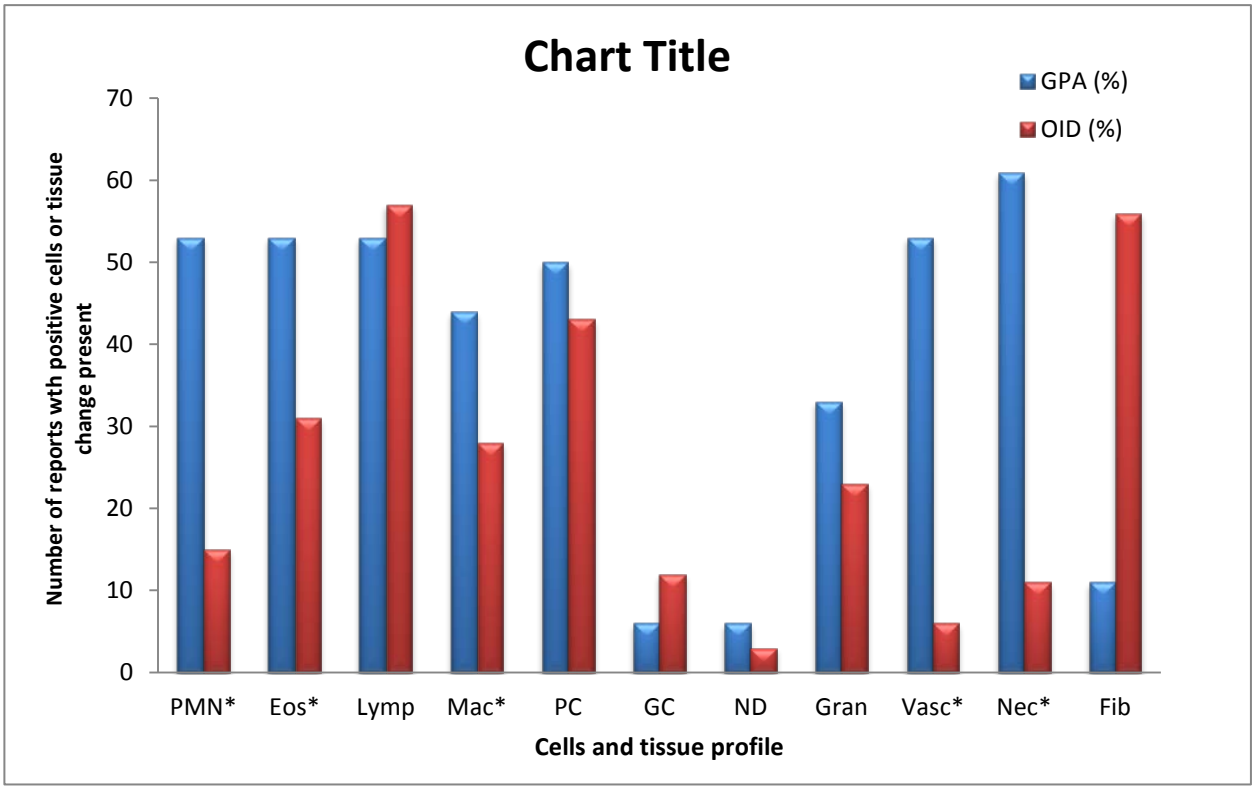
In the histopathology reports of GPA patients, the several inflammatory cells and tissue changes were cited more frequently more compared to the combined histopathology reports from OIDs. This indicated a more active and distinctive presence of inflammatory cells in GPA biopsies. (Table 3.1) (Figure 3.5)

Granulomatous inflammation was the predominant feature described for orbital GPA, as seen reported in all GPA biopsies. Comparisons of the cellular and tissue change occurrences reported as present in the histopathology report, between the GPA and non-GPA patients are summarised in Table 3.1. Neutrophil (p= <0.001), eosinophils (p=0.02), macrophages (p=0.04), vasculitis (p=<0.001), and necrosis (p=<0.001) were reported present significantly more in the GPA group compared to the OID group. Further multivariate analysis showed that the presence of neutrophils and vasculitis were independently associated with the clinical diagnosis of GPA with an odd ratio of 3.9 (p=0.01) and 4.8 (p=0.006) respectively (Table 3.1).

Table 3.1: Subjective comparison of the cellular and tissue change profile reported present in histopathology reports between GPA and OID (non-GPA) patients.

Cell type/tissue change	GPA (n=36)Total (%)	OID (n=198) Total (%)	Unadjusted		Adjusted	
			OR	p	OR	P
Neutrophils	19 (53%)	30.9 (15%)	5.9 (2.7-12.6)	<0.001*	3.9 (1.4-11.2)	0.01*
Eosinophils	19 (53%)	62 (31%)	2.5 (1.2-5.0)	0.02*	0.7 (0.24-1.8)	0.42
Lymphocytes	19 (53%)	113 (57%)	0.8 (0.4-1.7)	0.86		
Macrophages	16 (44%)	55 (28%)	2.1 (1.0-4.3)	0.04*	2.0 (0.9-4.5)	0.11
Plasma cells	18 (50%)	85 (43%)	1.3 (0.7-2.7)	0.47		
Giant cells	2 (6%)	23 (12%)	0.4 (0.1-2.0)	0.39		
Nuclear debris	2 (6%)	6 (3%)	1.9 (0.4-9.7)	0.36		
Granuloma	12 (33%)	45 (23%)	1.7 (0.8-3.7)	0.21		
Vasculitis	19 (53%)	11 (6%)	19.0 (7.8-46.4)	<0.001*	4.8 (1.6-14.7)	0.10
Necrosis	22 (61%)	21 (11%)	5.4 (2.4-12.0)	<0.001*	2.4 (0.9-4.5)	0.006*
Fibrosis	4 (11%)	111 (56%)	1.2 (0.6-2.6)	0.72		

OR = odds ratio; CI = confidence interval, % = percentage of patients with positive occurrence of cell type or tissue response reported in biopsy within each group, * = significantly different statistically ($p<0.05$).



PMN=neutrophil, Eos= eosinophils, Lymp=Lymphocytes, Mac=Macrophages, PC=plasma cells, GC=Giant cells, ND=Nuclear debris, Gran=granuloma; Vasc= vasculitis, Nec=Necrosis, Fib=Fibrosis, * = significantly different statistically ($p<0.05$).

Figure 3.5: Subjective comparison (via histopathology reports) of immune cells and inflammatory tissue changes between GPA and non GPA

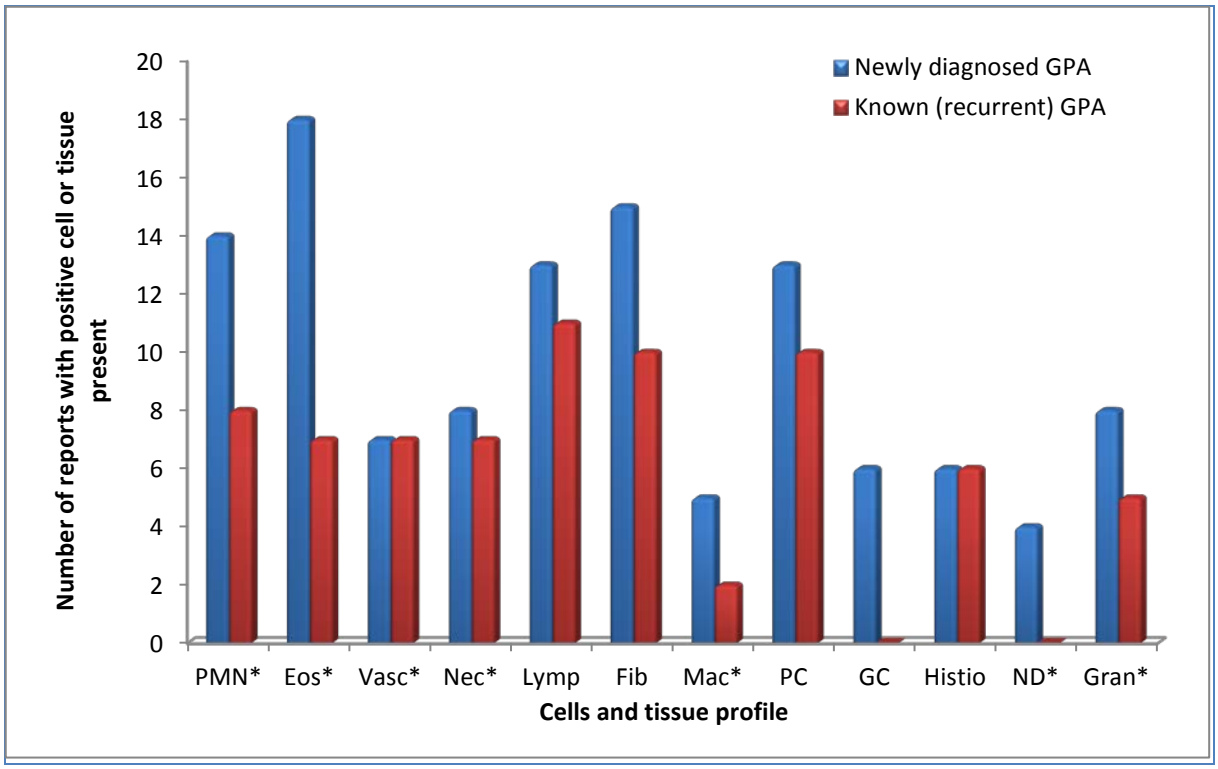
3.14.2 Subanalysis within the GPA Group

3.14.2.1 ANCA positive versus ANCA negative

Eosinophils and lymphocytes were more frequently observed in the ANCA positive group compared to the ANCA negative group. However these differences were found to be only borderline significant, with p values of 0.08 and 0.09, respectively.

3.14.2.2 Newly Diagnosed GPA versus Previously Known GPA

There were no significant differences in cellular occurrences between the newly diagnosed GPA and the known GPA patients. Nevertheless, there were more cellular activities observed in the newly diagnosed GPA group compared to the known GPA group. (Figure 3.6) Interestingly, nuclear debris and giant cells were not found in any of the biopsy of patients who are known GPA. Both entities were only found in the biopsies of those who were newly diagnosed with GPA.



PMN=neutrophil, Eos= eosinophils, Vasc= vasculitis, Nec=Necrosis, Lymp=Lymphocytes, Fib=Fibrosis, Mac=Macrophages, PC=plasma cells, GC=Giant cells, Histio=Histiocytes, ND=Nuclear debris, Gran=granuloma; * significantly different statistically ($p<0.05$)

Figure 3.6: Graph of cellular and tissue occurrence in biopsies between newly diagnosed GPA and those with orbital extension of pre-established GPA (known GPA). Newly diagnosed GPA shows more cellular activity compared to known GPA and nuclear dusts, and giant cells were reportedly seen only in newly diagnosed group.

3.15 Orbital Tissue Analysis (objective analysis)

3.15.1 GPA versus OID

A total of 239 tissue blocks from 234 patients were retrieved from thePathology Department, Institute of Ophthalmology, University College of London (UCL) archive storage. Out of these, 39 blocks were from patients diagnosed and clinically managed with GPA, and 200 blocks were from other orbital inflammatory diseases (OID) which included 65 IOID, 35 dacryoadenitis, 14 sarcoidosis, 12 thyroid eye diseases, four myositis, 23 lymphoid hyperplasia and 47 other OIDs such as dermoid, dacryosistitis, mucocele, orbital xanthogranuloma, Churgg Strauss syndrome and post-surgical granulomas.

The orbital biopsies showed a mixed picture of acute and chronic inflammation. Orbital biopsies from GPA patients were found to be heavily infiltrated with inflammatory cells resulting in an intense (darker) stained picture compared to other OIDs. (Figure 3.7)

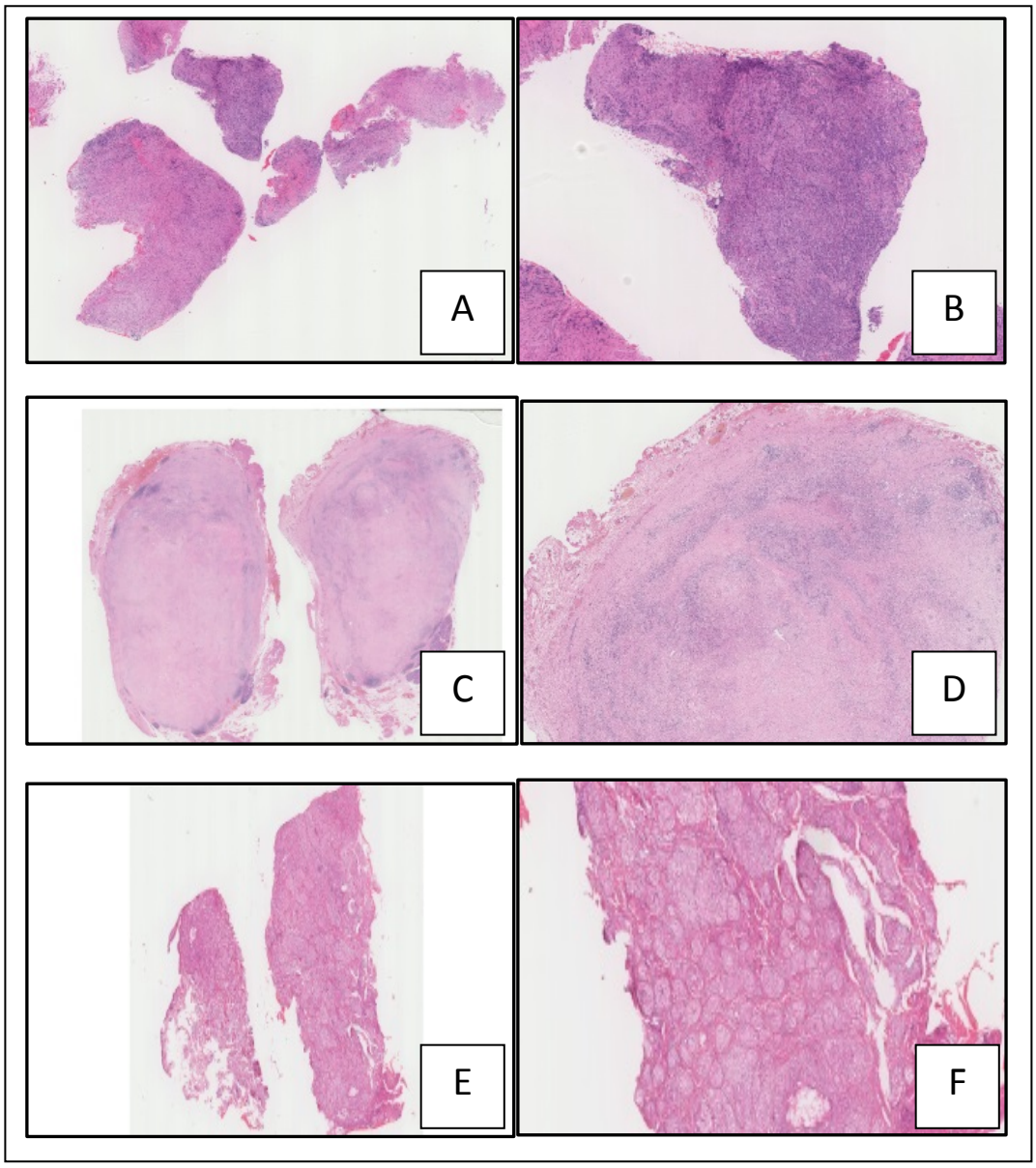


Figure 3.7: H&E stained tissue biopsies. All tissues were stained in parallel i.e. at the same time and with the same technique and laboratory condition. A, C and E are at 40X magnification and B,D and F are at 100X magnification.(A&B) represent H&E stained tissue biopsy of GPA showing a more infiltrated, densly packed and heavily stained picture compared to tissue biopsy from IIOD (C&D) and orbital sarcoidosis (E&F)

Objective count comparisons for each cell types and tissue changes are summarised in Table 3.2. Further multivariate analysis showed that necrosis and vasculitis were independently associated with the clinical diagnosis of GPA with an odd ratio of 2.40 ($p<0.001$) and 1.33 ($p<0.001$) respectively. However, not all slides from this GPA group had features of necrosis and vasculitis. 12 out of 39 orbital GPA biopsies (31%) did not have necrosis and five (13%) from these did not have both vasculitis and necrosis. Interestingly, granuloma was shown to be significantly less in orbital GPA biopsies compared to OIDs, and in addition, it was found to be inversely associated with the clinical diagnosis of orbital GPA (odds ratio = 0.12, $p=0.02$). (Table 3.2)

Table 3.2: Quantitative (objective count on H&E slides) Cell/Tissue Comparison between GPA and other OIDs. Cell count were performed in 20x1mm^2 (20mm^2) field in each case and tissue change were determined and counted from 20 random points generated

Cell & tissue changes	GPA (n=39) Mean +/- SE	Other OIDs (n=200) Mean +/- SE	Unadjusted	Adjusted	
			p	OR	*p*
Neutrophils	37.2 +/- 10.4	11.1 +/- 2.4	<0.001 *	0.99 (0.97 - 1.01)	0.26
Eosinophil	50.0 +/- 19.4	12.4 +/- 2.4	0.002 *	1.00 (0.99 - 1.01)	0.50
Lymphocyte	3292.3 +/- 141.2	3671.5 +/-187.6	0.582		
Macrophage	290.5 +/- 19.7	188.89 +/- 8.8	<0.001 *	1.00 (0.99 - 1.01)	0.34
Plasma cells	81.82 +/- 16.5	66.30 +/- 6.4	0.146		
Giant cells	0.26 +/- 0.9	0.32 +/- 0.1	0.066		
Mast cells	0.33 +/- 0.2	0.12 +/- 0.03	0.096		
Nuclear debris	3.7 +/- 1.5	0.96 +/- 0.3	0.011 *	1.08 (0.96 - 1.22)	0.19
Granuloma	0.5 +/- 0.4	0.55 +/- 0.1	0.046 *	0.12 (0.21 - 0.71)	0.02*
Vasculitis	7.13 +/- 0.8	2.95 +/- 0.2	<0.001 *	1.33 (1.15 - 1.54)	<0.001 *
Necrosis	3.0 +/- 0.4	0.35 +/- 0.75	<0.001 *	2.39 (1.73 - 3.3)	<0.001 *
Fibrosis	6.59 +/- 0.5	7.63 +/- 0.5	0.906		

OR = odds ratio; CI = confidence interval, * = significantly different statistically ($p<0.05$)

3.15.2 **Subanalysis within the Orbital GPA Group**

3.15.2.1 Newly Diagnosed GPA versus Known GPA

18 out of 39 blocks from the GPA group were newly diagnosed GPA while the remaining 21 were previously diagnosed with and treated for GPA from other clinical manifestations before their orbital presentation i.e. known GPA. Comparison between these groups showed that eosinophils (p=0.045) and macrophages (p=0.06) were found marginally more in the known GPA group compared to the newly diagnosed GPA group. (Figure 3.6)

3.15.2.2 Other Comparisons in the GPA Group

There were no differences in any of the cell and tissue counts with regards to ANCA status (positive versus negative) (p=0.1), disease extension (generalised versus localised GPA) (p=0.3) and treatment regime (single or multiple therapies) (p=0.1).

3.16 Discussion

In this part of the study, our main aim was to see if there is a difference between the histology of orbital GPA and OIDs, by comparing the cell types and tissue changes subjectively (via histopathology reports) and objectively (via immune cell and tissue change counts).

We found that even based on only histopathological reports; there were differences in the cellular activity between orbital GPA and other OIDs where GPA portraying a more active cellular pattern in their biopsies. The same findings were also true when we performed objective cellular and tissue change comparison on H&E stained tissue analysis. Tissue biopsies of GPA patients were found to have increased immune cell activity compared to the other OIDs combined. This is reflects that ocular GPA can have a more aggressive clinical course compared to other OIDs, and can cause severe ocular morbidity. The appearance of the H&E stained tissues of orbital GPA displayed a busier, deeply stained and mixed cellular composition compared to other OIDs. This appearance is consistent with the histology of lung tissues affected with GPA; described by one article asareas with a deep basophilic, “dirty” appearance which is suggested to be due to the high presence of neutrophils and nuclear debris (Lagstein and Myers, 2009)(Mukhopadhyay and Gal, 2010).

Vasculitis and necrosis were features independently associated with the diagnosis of orbital GPA in this study. These two features are in keeping with the classic triad of the pathological features of GPA which are granulomatous inflammation, vasculitis and extensive tissue necrosis. Vasculitis in particular, is said to be an important feature in the histology of GPA where one study suggested that, granulomatous inflammation without vasculitis is more characteristic of other orbital inflammatory diseases rather than GPA (Bijlsma et al. 2011). In the lungs, necrotizing vasculitis is said to be the single most important feature in the histological diagnosis of GPA (Mukhopadhyay and Gal, 2010). Similarly, in the kidney, the main histological feature seen in patients with active renal GPA is segmental glomerular necrosis (Aasarød et al., 2001a)(Aasarød et al., 2001a). Together with the higher inflammatory activity observed, it is likely that the presence of necrosis and vasculitis seen clinically, reflect the more severe and destructive nature of orbital GPA, compared to other OIDs. Nevertheless, it is important to point out that in a number of the

orbital GPA tissues in our study, features of necrosis and vasculitis were absent despite being at the point of active inflammation. This may largely be attributed to the small tissue sample.

GPA and sarcoidosis are both described as granulomatous inflammatory diseases. However, the histology of sarcoidosis differs slightly compared to GPA where in sarcoidosis, features of discrete, well-formed, interstitial non-necrotizing granulomas are mainly seen (Mukhopadhyay and Gal, 2010). In GPA, the term “granulomatous inflammation” described mainly made up of loose collections of histiocytes, multinucleated giant cells and other inflammatory cells, is said to be poorly defined (Holle et al., 2010a)(Holle et al., 2010a). Even in the lungs, compact, ‘sarcoid-like’, non-necrotizing granulomas are said to be exceptional in GPA (Travis et al., 1991). Granulomas in the lungs with GPA are suppurative or neutrophil filled in nature, with a dirty appearance due to the high presence of neutrophil and nuclear debris. They are also described to have an irregular contour, unlike granulomas seen in sarcoid or infection (Mukhopadhyay and Gal, 2010). The histology of our orbital GPA biopsies also showed an inflammatory picture with abundance of macrophages where distinct granuloma formations were very much less.

We also observed a high count in neutrophils and nuclear debris in orbital GPA biopsies. Neutrophil in particular, when analysed based on histopathology reports, were found to be independently associated with the diagnosis of orbital GPA. In addition, nuclear debris, which is thought to originate from fragmented neutrophils, have been repeatedly described in pathological reports in GPA (Ahmed et al., 2008)(Lamprecht and Gross, 2007)(Ahmed et al., 2008), but does not seem to be observed in the biopsies of any other ocular inflammatory conditions. Nuclear debris is an entity that has only recently become a

subject of interest in GPA. In our study, the occurrence of nuclear debris reported in histopathology reports in the whole sample, were most likely to be under-represented as pathologists may not have specifically reported this phenomenon in the past. Nevertheless, despite this, the presence of nuclear debris was still reported more in the GPA group compared to other OIDs. This finding was also consistent in the H&E tissue analysis.

ANCA has been demonstrated to play a considerable role in the pathogenesis of vasculitides. The presence of ANCA causes increased neutrophil migration, and adhesion to vascular endothelium and subsequent neutrophil degranulation leading to vascular inflammation or vasculitis. This may explain the presence of nuclear debris in biopsies of GPA in this study but not in the biopsies of other orbital inflammatory diseases which are not associated with ANCA. The exact aetiology of ANCA production and why it is involved in AAV still remains unclear. One theory suggests that the production of ANCA may be related to ineffective neutrophil apoptosis (programmed cell death) or ineffective removal of the apoptotic cell fragments of neutrophils. Therefore, alternatively, the presence of these nuclear debris, that are thought to originate from neutrophils may be triggering stimuli for the production of ANCA instead. This may also explain how ANCA, which targets neutrophil intracellular antigens, may be raised in these diseases. In addition, on examining the histopathology reports, GPA subgroup analysis showed that nuclear debris were only reported in the newly diagnosed patients which strongly suggests the active role of neutrophils in the acute and initial stage of the disease.

The role of eosinophils in the pathogenesis of GPA is not clear although its presence in orbital GPA biopsies has been documented in several publications (Ahmed et al.,

2008)(Choopong et al., 2005). In this study, eosinophils were found to be significantly higher in orbital GPA compared to other OIDs although this was lost when other confounding factors were controlled for. This suggests that tissue eosinophils in OIDs might be associated with disease severity, rather than being associated with GPA in particular. In addition, based on our H&E analysis, eosinophils were observed to be marginally more in patients with known GPA compared to newly diagnosed GPA. This may indicate two important possibilities: (1) that recruitment of eosinophils are higher during the recurrence of GPA, or (2) that eosinophils are unaffected by treatment and are retained in the affected peripheral tissues. Thus, apart from being associated with disease severity in OIDs, the role of eosinophils in orbital GPA may also be associated with disease recurrence or chronicity.

Lymphocyte count was overall markedly higher compared to other immune cells. Lymphocytes are one of the major effector cells in the inflammatory response and play a role in both the acute and chronic inflammatory processes. Lymphocytes can generally be T cells, B cells or natural killer cells (NK T cells), where each subtypes have specific important roles in the immune system. T cells and B cells in particular are responsible for the cellular and adaptive immune responses respectively; both critical human defense systems. In the active state, lymphocytes also help recruit other immune cells and biochemical entities to the target site further perpetuating the immune response. Over activity of these immune cells as well as other cells in the inflammatory process is the basis of chronic inflammation. Lymphocytes subtypes have been linked to the pathogenesis of GPA however in H&E tissues, it is not possible to distinguish these subtypes apart. This could only be done with immunohistochemistry staining.

We also performed subgroup histology comparison between biopsies of newly diagnosed GPA and known GPA patients. Although there were no significant differences seen in the occurrence of cells and tissue changes, newly diagnosed GPA appeared to have more cellular activity compared to known GPA. Also, as previously mentioned, nuclear debris as well as giant cells were found only in patients who were newly diagnosed with GPA. This observation may explain the findings in the first part of our study where we noted that newly diagnosed patients tend to present more severe clinical symptoms compared to known GPA. This outcome is also comparable to a previous study by Stone JH in 2003 who reported that in patients with GPA, the disease was more severe in the newly diagnosed GPA compared to relapse cases (Stone, 2003). Therefore, these results appear to suggest that in orbital GPA, (1) cellular activity is more intense in the first episode of the disease, (2) the cellular activities change as inflammation in GPA becomes chronic or when the disease relapses, and (3) treatment may alter the cellular profile or activity in GPA.

3.17 Conclusion and Further Plans

There were no distinctive features found in the biopsies of orbital GPA that could act as a marker for early diagnosis. We found that there are significant differences observed in the levels of cellular activity between GPA orbital biopsies and OIDs where orbital GPA had significantly higher inflammatory activities compared to OIDs. This mirrors the more severe clinical presentation of orbital GPA. The cause of this increased activity is still unknown and factors that enhance the recruitment of these inflammatory cells are also unclear.

It is interesting to note that there were also differences found in the cellular activities between orbital biopsies of newly diagnosed GPA patients compared to orbital biopsies of

known GPA patients. This may suggest that in GPA, cellular activities in the peripheral tissues may be influenced by disease chronicity and treatment.

The main difference in the histology of orbital GPA, as already been well established by previous studies, are the prominent presence of necrosis and vasculitis i.e. the key histological features in the diagnosis of GPA. However, these features were not always present in tissue biopsies of patients with GPA, as discovered in this study. It is also important to note that there were also significant differences found in the presence of certain cells such as neutrophils and macrophages. In addition, it was also interesting to discover that granuloma formation was inversely associated in the biopsies of orbital GPA despite the disease being described as a granulomatous inflammatory disease. This is a marked contrast from orbital sarcoidosis which is also described as a granulomatous inflammatory disease where granuloma formations in tissues are the key feature. Thus from these findings, there may be a possibility of specific sub-types of the inflammatory cells present in orbital GPA that differ from other OIDs, that could account for the difference in the clinical manifestation in orbital GPA compared to other OIDs.

We therefore proceeded to further investigate this with immunohistochemical staining and looked for specific cell sub-types, cell receptors and cytokines from the different infiltrating cells such as T lymphocytes, B lymphocytes, macrophages and neutrophils in orbital GPA biopsies.

We chose to compare the presence of these cells specifically with two other OIDs which are orbital sarcoidosis and IIOD. The purpose for this is because IIOD shares very similar disease presentation and progression with orbital GPA and is often very difficult to discern from orbital GPA clinically, and orbital sarcoidosis has the same inflammatory process as orbital GPA i.e. granulomatous inflammation although it does not usually share similar clinical progression and outcomes with orbital GPA.

4 Chapter 4: T lymphocytes

4.1 T cells in GPA

The involvement of T lymphocytes (T cells) in the pathogenesis of GPA has been well documented (Hua et al., 2009)(Lamprecht and Kabelitz, 2011)(Popa et al., 1999). In tissue biopsies of GPA, T cells and macrophages are seen to predominate. However, total blood lymphocyte numbers, and absolute and relative numbers of CD4 T helper cells were observed to be relatively low in GPA patients compared with control samples. It is postulated that this lymphopenic effect in GPA may occur due to extensive migration of blood T cells to tissues, and also as a result of treatment effects (Berden et al., 2009). T cells are seen in abundance in kidneys of patients with crescentic glomerulonephritis and correlate negatively with renal function (Aasarød et al., 2001b)(Aasarod K 2001).

4.2 CD4 T cells in GPA

CD4 T cells and its various subsets have been shown to have a significant role in the development of the disease in GPA (Lamprecht and Kabelitz, 2011)(Abdulahad et al., 2006)(de Menthon et al., 2011) (Abdulahad et al 2006). Patients with GPA appear to have an increased CD4+ effector memory T cells compared to naïve CD4 cell population during remission. However, during disease activity, serum effector memory T cells are found to decrease, indicating recruitment of these cells into affected tissues (Abdulahad et al., 2006). Indeed, CD4+ effector memory cells have been found in urine of patients with active glomerulonephritis secondary to GPA (Abdulahad et al., 2009). Tregs have also been shown to be defective in GPA that may be responsible for the relapsing and remitting behaviour of the disease (Abdulahad et al., 2007). Nonetheless, the cause of the dysfunction in these cells in GPA is still unclear. Other effector CD4 cells such as

CD4+CD28- cytotoxic T cell, have also been seen to be expanded in GPA and to do so even during disease remission (Abdulahad et al., 2006).

4.3 Th1 T cells and Th2 T cells in GPA

Both Th1 T cells and Th2 T cells have been implicated in the disease development in GPA. A study by Csernok E in 1999 showed biopsy of nasal granuloma in GPA expressed IFN- γ with no IL-4, indicating a strong Th1 T cells link (Csernok et al., 1999). However, a similar study, also with GPA nasal biopsies by Balding et al in 2001, revealed that nasal tissue from patients with GPA showed an increased expression of IL-4, down-regulation of IL-2 and no detectable IFN-gamma, thus implicating a Th2 T cells response (Balding et al., 2001). It has been suggested that Th1 T cells may play a role in localised GPA and as the disease progresses to become systemic, a shift to a Th2 T cells response may occur. This however has yet to be confirmed (Berden et al., 2009).

4.4 Th17 in GPA

The role of T-helper 17 (Th17) cells have recently been investigated as potential players in disease development in AAV, including GPA (Berden et al., 2009). Th17 has also been reported to be instrumental in the pathogenesis of these diseases (Kallenberg, 2011a). In particular, levels of IL-17 were found to be high in patients with GPA compared to healthy individuals, and in ANCA positive GPA patients, Th17 was found to be skewed upon stimulation of PR3 (Abdulahad et al., 2008). Th17 is also seen to be expanded in both active and quiescent GPA (Wilde et al., 2012).

4.5 CD8 in GPA

CD8 as well as CD4 have been shown to be activated in GPA (Schlesier M 1995). CD8 T cell sub-types such as CD8+CD57+ (Iking-Konert et al., 2009) have been implicated in the pathogenesis of the disease and is said that it could have a direct contribution to vascular endothelium damage seen in the disease (Schlesier et al., 1995). CD8 has also been found in tissues of organs affected by GPA. In kidneys affected with GPA, CD8 (cytotoxic) T cells were found to be present in the intraglomerular tissues where these cells made up two-thirds of the total T cells in the tissue. In particular, not only CD8 T cells together with macrophages were found to be the predominant cellular infiltrates in these tissue biopsies, it is suggested that CD8 also carries a prognostic value where the presence of these cells in renal tissues indicate a poorer renal prognosis (Aasarød et al., 2001a)(Aasarød et al., 2001a).

4.6 Other T cell subtypes in GPA

Other T cell sub-types reported include the presence of CD134 in various organ tissue biopsies affected by GPA. CD134 has been demonstrated to be present in renal, lungs and nasal tissues affected by GPA (Wilde et al., 2009). CD134, also known as OX40, is a member of the tumour necrosis factor receptor (TNFR)-superfamily and a co-stimulatory molecule. The expression of CD134 is dependent on full activation of T cells, thus are not expressed on naïve T cells. It plays a critical role in maintaining an immune response by preventing activated T cell death. In GPA, CD134 is not only postulated to have a role in the disease development, but is also said to be responsible for the relapsing disease course where circulating CD134 subsets is thought to be the starting point for organ and tissue inflammation (Wilde et al., 2009).

4.7 T cells in sarcoidosis

CD4 T cells play an important role in the pathogenesis of sarcoidosis. In target organ sites, granuloma formation; classically seen in the histology of sarcoidosis, is driven by the CD4, Th1 T cells, macrophage and INF-γ production immunologic response (Grunewald and Eklund, 2007)(Grunewald et al., 2006). Indeed, lymphopenia involving CD4, CD8 and CD19 has been observed in patients with sarcoidosis, indicating recruitment into active target organs. The degree of lymphopenia was also seen to correlate with the degree of disease activity (Sweiss et al., 2010). The use of bronchoalveolar lavage fluid (BALF), looking at CD4/CD8 ratios in the lungs, is currently widely used in the diagnosis of sarcoidosis. It is said that the ratio of CD4/CD8 of 2 and more indicate a strong positive diagnosis for sarcoidosis (Winterbauer et al., 1993). In the eye, CD4/CD8 ratios in vitreous samples were also shown to have similar findings with BALF in patients with sarcoidosis, implying a high diagnostic value in the diagnosis of ocular sarcoidosis (Kojima et al., 2012). Apart from this, Th17 has also been proposed to have a role in the pathogenesis of sarcoidosis. An imbalance in Th17 and Tregs in peripheral blood and BALF has been associated with the diagnosis of sarcoidosis (Huang et al., 2013).

4.7.1 T cells in IIOD

There have not been many reports or investigations performed to look into the role of T cells in the pathogenesis of IIOD. However, it has been shown that in the histology of IIOD, a predominance of T cells were seen when compared to lymphoid hyperplasia which had a mixed T and B lymphocytes, and lymphoma which had predominant B cell involvement (Lowen et al., 2005). The treatment of refractory IIOD does include medication with T cell

and B cells immunosuppressor activity such as mycophenolate mofetil, thus indicating the active role of these cells in this disease (Patel et al., 2011).

4.8 Objective

We wanted to demonstrate whether the T cells sub-types seen in serum and other organs affected by GPA were also present in GPA orbital tissues. In particular, we wanted to compare the presence of these cells to IIOD and orbital sarcoidosis to discover the significant role of these cells in the disease process of orbital GPA. The main objectives for this part of the study are:

1. To demonstrate the presence of CD3, CD4, CD8, CD134 and cytokine IL-17 in orbital tissue biopsies of GPA,
2. To compare the presence of CD3, CD4, CD8, CD134 and cytokine IL-17 between orbital tissue biopsies of GPA and IIOD, and
3. To compare the presence of CD3, CD4, CD8, CD134 and cytokine IL-17 between orbital tissue biopsies of GPA and orbital sarcoidosis

4.9 Method

4.9.1 Subject and Tissue selection

Paraffin blocks of 25 patients from the orbital GPA group and 25 patients from the IIOD group were randomly selected from the pool of patients already included for the previous section of the study. Due to insufficient tissue in one case, only 13 paraffin blocks from the 14 patients clinically diagnosed with orbital sarcoidosis were used for the IHC study.

4.9.2 Antibodies

IHC was performed for cell markers CD3 (A0452, DAKO, Cambridgeshire, UK) protein complex, CD4 (Clone 4B12; M7310, DAKO, Cambridgeshire, UK), CD8 (Clone C8/144B; M7103, DAKO, Cambridgeshire, UK); CD134 (Clone ACT35; 555836, BD Pharmingen™, Becton, Dickinson and Company, Oxford, UK) and IL-17A (IL-17A; AHP455G AbDSerotec, Kidlington, UK).

4.9.3 Slide preparation

Slide preparations and stainings for each antibody were performed on the same day and under the same laboratory conditions for all cases. Tissue sections from the blocks were cut using a sledge microtome at a thickness of 5 micrometer and mounted on Superfrost-plus object glass slides. The slides were left on a hot plate at 40°C for 60 minutes and then incubated overnight at 37°C before use.

4.9.4 **Immunohistochemistry staining of fixed paraffin tissues (Table 4.1)**

Slide preparations and stainings for each antibody were performed on the same day and under the same laboratory conditions for all cases. Tissue sections were de-waxed in two changes of xylene (each 5 to 10 minutes) with agitation at regular intervals, then twice with 100% ethanol (10 to 20 seconds) and then hydrated in 90%, 70%, and 50% ethanol, each for 10 to 20 seconds, followed by two washes in water. Sections were then incubated in the appropriate antigen retrieval solution where Tris/EDTA pH 9.0 (H-3301, Vector Labs, UK) was used for CD3, CD4, CD8, and sodium citrate pH 6.0 (H-3300, Vector Labs, UK) was used for CD134 and IL-17A. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes.

Table 4.1: Immunohistochemistry protocol for T cells investigations

Cell type	Antigen retrieval	Epitope retrieval	Mode of staining	Antibody concentration	Incubation time (temperature)	Result
CD3	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Autostainer	1:400	_	Positive
CD4	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Autostainer	1:50	_	Positive
CD8	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Autostainer	1:50	_	Positive
CD134	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours (4^{o}C)	Positive
IL-17A	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:800	24 hours (4^{o}C)	Positive

4.9.5 **Automated staining for CD3, CD4 and CD8**

CD3, CD4 and CD8 stainings were then performed by the DAKO Autostainer Plus on all slides at concentrations of 1:400, 1:50 and 1:50 respectively as per manufacturer recommendation. Positive and negative controls with inflamed tonsil tissue were also performed.

4.9.6 **Manual staining for CD134 and IL-17A**

Tissue staining for CD134 and IL-17A were stained manually; concentrations for both primary antibodies used were titrated using inflamed tonsil tissues until an optimal tissue stain were achieved.

After de-waxing and antigen retrieval as previously described above, slides were placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdgePen H-4000). Primary antibodies; CD134 (1:200) and IL-17A (1:800), diluted in blocking solution were added and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit K5005 AP/RED, Rabbit/Mouse; DAKO, UK was used forCD134 and IL-17A visualization. Finally, the slides were counter-stained with haematoxylin, then mounted with DPX mounting medium and cover slips, and allowed to dry overnight before imaging. Positive and negative controls with inflamed appendix tissue for each antibody were also performed.

4.10 Imaging and image analysis

4.10.1 IHC

ADCIS ® Stereology Toolkit frame count programme was used as before. Patient's identity and the diagnosis for each slide were masked during the counting process. For IHC, a total of 50 count frames (instead of 20) were randomly generated by the software for all slide images making up a total area of 50 mm^2 analysed for every slide. As previously done, only stained cells within the frame and not touching the exclusion lines were included in the count, and the software then generated the total number of marked cells to generate the total count.

4.10.2 Validation process for cell and tissue counts

Identification of cells and tissue changes were counter checked and validated by a senior pathologist (secondary supervisor) who was also masked from patients' identities and diagnoses. A portion of the slides were also re-counted to ensure total counts were reproducible.

4.10.3 Data analysis

Cell counts were entered into an Excel spreadsheet. The Mann Whitney test was performed with SPSS17 for quantitative comparisons of the individual stained cells between GPA and IIOD, and GPA and orbital sarcoidosis. Odds ratio was calculated via the 2x2 frequency table.

4.11 Results

4.11.1 T cell identification in orbital GPA, IIOD and sarcoidosis

CD3, CD4, CD8, CD134 and IL-17A positive stained cells were found present in orbital GPA tissue biopsies as well as in tissue biopsies of sarcoidosis and IIOD.

4.11.2 Cell count comparison between GPA and IOID

Count comparison for each T cell sub-types between GPA and IIOD are summarised in Table 4.2 and Figure 4.1– Figure 4.5. T cells (CD3) ($p=0.01$, $OR=1.57$) and CD8 ($p=0.04$, $OR=1.56$) were found significantly more in orbital GPA when compared with IOID. Other T cell sub-sets such as CD4 and CD134 were found to be comparable between the two diseases. In particular, IL-17A cytokine expression in orbital GPA was found to be considerably higher in GPA compared to IOID ($p<0.001$) and further odds ratio calculation revealed that a high IL-17A count in an orbital biopsy was likely to be from GPA tissues and not from IIOD ($OR= \infty$).

Table 4.2 Quantitative T-Lymphocyte, T- Lymphocyte Subsets and Cytokines cell counts and comparisons between orbital GPA and IIOD (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA (mean +/-SEM)	IOID (mean+/-SEM)	p	OR	CI
CD3	5221+/-449 (130532)	3633+/-560 (90827)	0.01*	1.57	0.24 -10.30
CD4	1157+/-145 (28914)	864+/-162 (21606)	0.07		
CD8	1041+/-140 (26021)	764.5+/-15.1 (19113)	0.04*	1.56	0.24 -10.30
CD134	94.6+/-18 (2365)	59+/-11 (1475)	0.09		
IL-17A	270+/-39 (6758)	88+/-9 (2212)	<0.001*	∞*	∞

* = significant odds ratio

4.11.3 **Cell count comparison between GPA and sarcoidosis**

Count comparison for each T cell sub-types between GPA and sarcoidosis are summarised in Table 4.3 and Figure 4.1 - Figure 4.5. CD3, CD4, CD8 and CD134 counts were found to be comparable between these two diseases. However, marked IL-17A expressions were observed in orbital GPA compared to sarcoidosis (p=<0.001). Further odds ratio calculations revealed that a high IL-17A count in an orbital biopsy was more likely to be from GPA tissues, and not from orbital sarcoidosis (OR= ∞).

Table 4.3 Quantitative T-Lymphocyte, T- Lymphocyte Subsets and Cytokines cell counts and comparisons between orbital GPA and orbital sarcoidosis (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA (mean+/-SEM)	Orbital sarcoidosis (mean+/-SEM)	p	OR	CI
CD3	5221+/-449	4951+/-851 (64369)	0.5		
CD4	1157+/-145 (28914)	955+/-198 (12422)	0.5		
CD8	1041+/-140 (26021)	1015+/-267 (13200)	0.3		
CD134	94.6+/-18 (2365)	150+/-37 (1955)	0.7		
IL-17A	270+/-39 (6758)	71 +/-16 (924)	<0.001*	∞*	∞

* = significant odds ratio

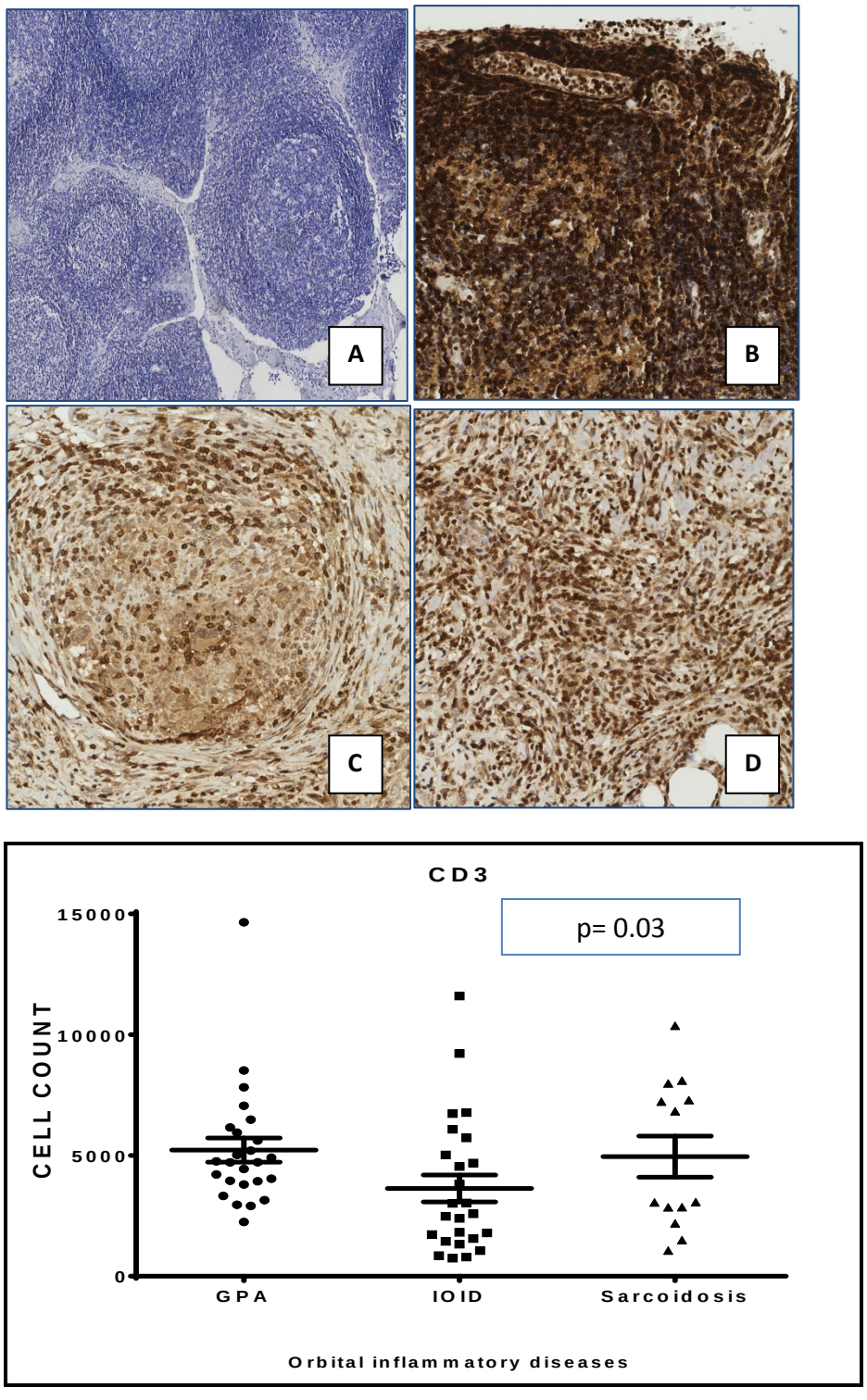


Figure 4.1: CD3 tissue stainings. A: Negative CD3 staining on an inflammed tonsil (primary antibody not added, x100), B: CD3 staining on a GPA orbital tissue (HRP/DAB+, Rabbit/Mouse; x100), C: CD3 staining on an IIOD (HRP/DAB+, Rabbit/Mouse; x100), D: CD3 staining on an orbital sarcoidosis tissue. (HRP/DAB+, Rabbit/Mouse; x100), E: Scatter plot of CD3 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing significant difference between them (p<0.03). Dunn's multiple comparison revealing the main difference in CD3 cell counts between and GPA and IOID.

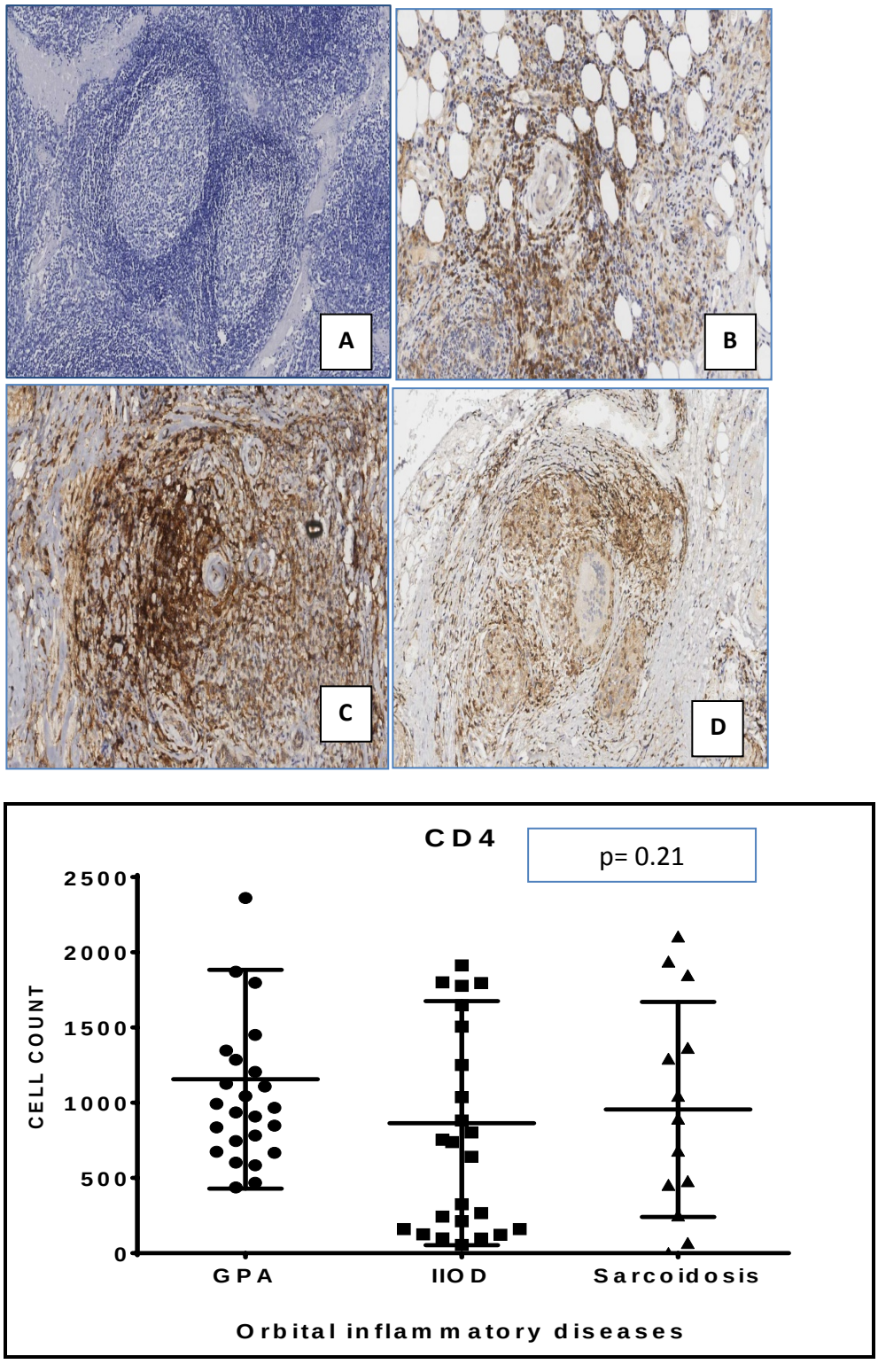


Figure 4.2: CD4 Tissue stainings A: Negative CD4 staining on an inflammed tonsil (primary antibody not added, x100), B: CD4 staining on a GPA orbital tissue . (HRP/DAB+, Rabbit/Mouse; x40), C: CD4 staining on an IIOD tissue (HRP/DAB+, Rabbit/Mouse; x40), D: CD4 staining on an orbital sarcoidosis tissue (HRP/DAB+, Rabbit/Mouse; x40), E: Scatter plot of CD4 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing no significant difference between them (p<0.21).

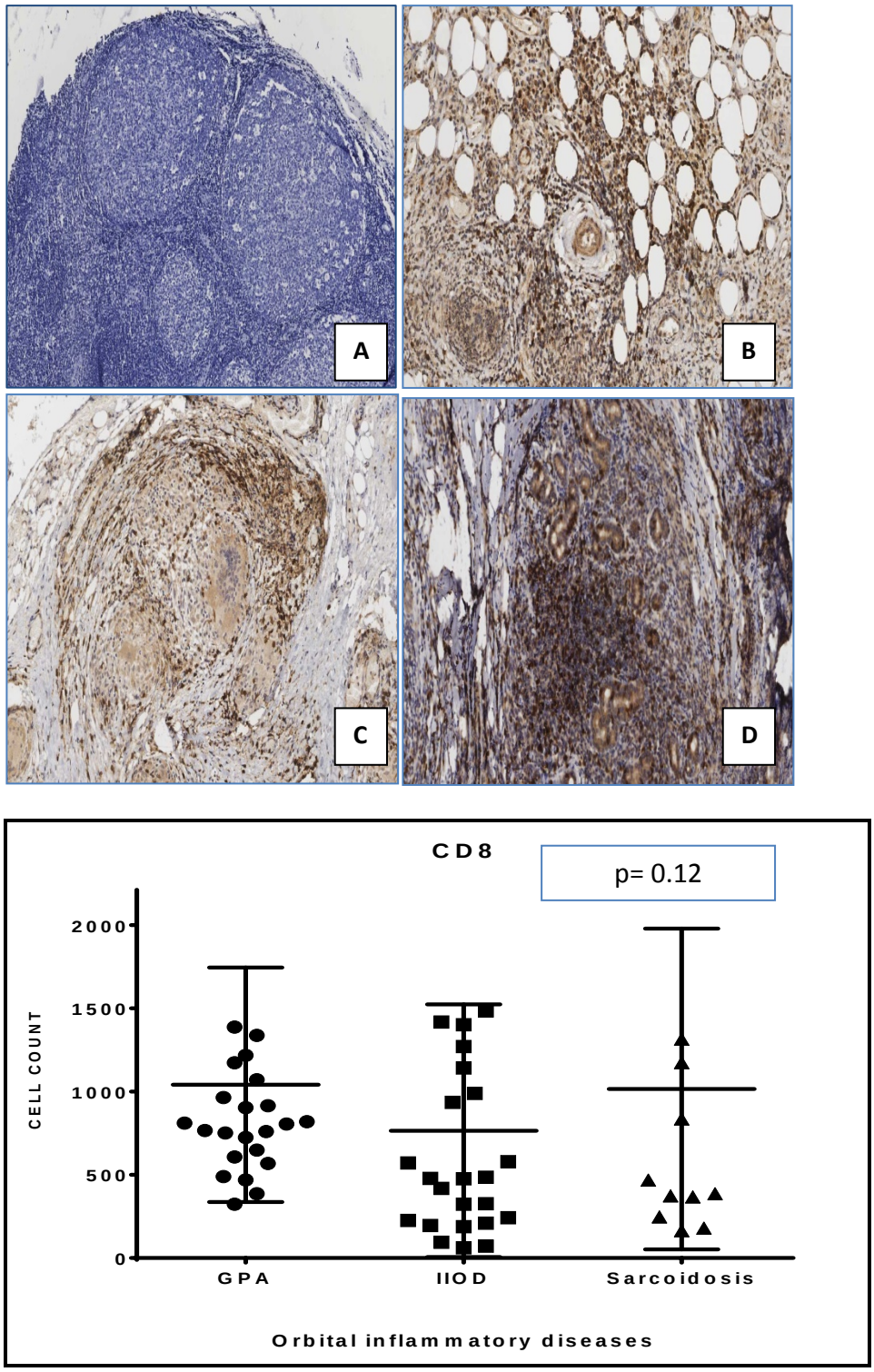
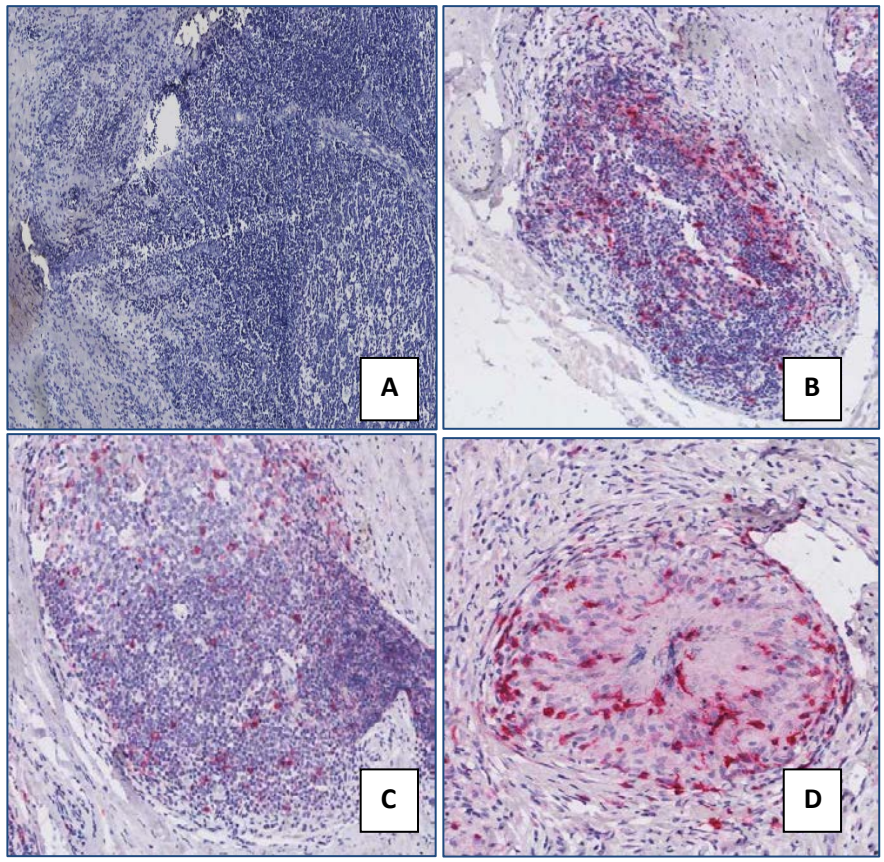


Figure 4.3: CD8 tissue stainings with HRP/DAB+ A: Negative CD8 staining on an inflammed tonsil (primary antibody not added, x100), B: CD8 staining on a GPA orbital tissue, (HRP/DAB+, Rabbit/Mouse; x40), C: CD8 staining on an IIOD tissue (HRP/DAB+, Rabbit/Mouse; x40), D: CD8 staining on an orbital sarcoidosis tissue (HRP/DAB+, Rabbit/Mouse; x40), E: Scatter plot of CD8 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing no significant difference between them (p<0.12).



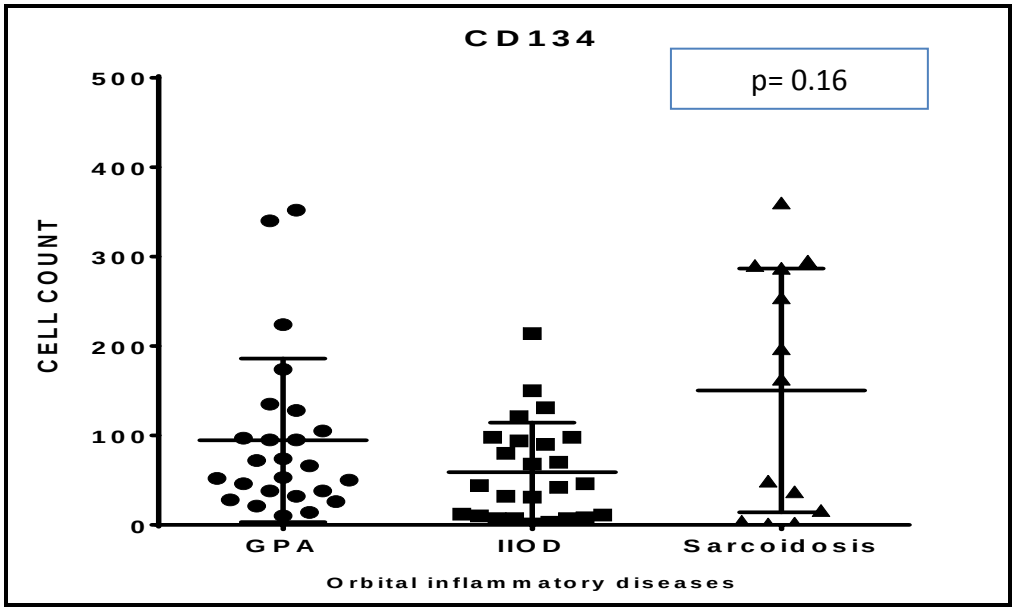


Figure 4.4: CD134 tissue stainnigs with AP/RED A: Negative CD134 staining on an inflammed tonsil (primary antibody not added, x100), B: CD134 staining on a GPA orbital tissue. (AP/RED, Rabbit/Mouse; x40), C: CD134 staining on an IIOD tissue (AP/RED, Rabbit/Mouse; x40), D: CD134 staining on an orbital sarcoidosis tissue (AP/RED, Rabbit/Mouse; x40), E: : Scatter plot of CD134 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing no significant difference between them (p<0.16).

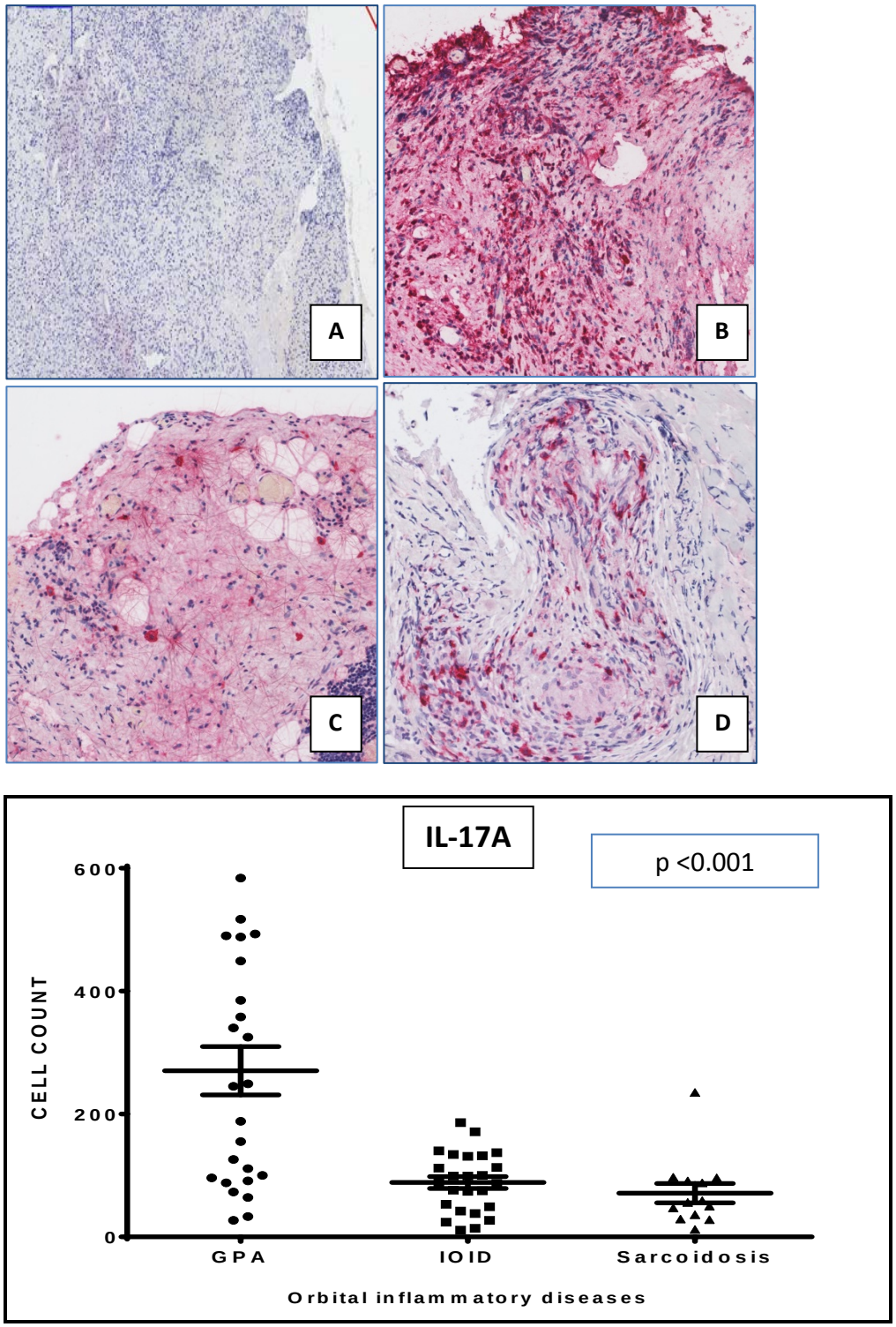


Figure 4.5: IL-17A tissue stainings with AP/RED A: Negative IL-17A staining on an inflammed tonsil (primary antibody not added, x100), B: IL-17 staining on a GPA orbital tissue (AP/RED, Rabbit/Mouse; x100), C: IL-17A staining on an IIOD (AP/RED, Rabbit/Mouse; x100), D: IL-17A staining on an orbital sarcoidosis tissue. (AP/RED, Rabbit/Mouse; x100), E: Scatter plot of IL-17A showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing significant difference between them (p<0.001). Dunn's multiple comparison revealing IL-17A significantly more in GPA compared to both IOID and orbital sarcoidosis.

4.12 Discussion

In this section of the study, we were able to demonstrate the presence of CD3, CD4, CD8, CD134 and IL-17A in orbital GPA, sarcoidosis and IIOD tissues.

The presence of T cells in general, found in all GPA, IIOD and sarcoidosis biopsies were consistent with previous reported studies demonstrating an important role of this cell in each of these orbital diseases. In this study, we further showed that the presence of CD3, CD4, and CD8 were comparable between GPA, IIOD and sarcoidosis implying that these immune cells have equal importance in the pathogenesis of these diseases.

The IL-17A cytokine findings in our orbital GPA were particularly significant. IL-17A is now shown to be present in the disease process in orbital GPA like other autoimmune inflammatory diseases, such as rheumatoid arthritis and SLE. More importantly, the level of IL-17A in our orbital biopsies was markedly more in the orbital GPA compared to IOID and sarcoidosis, suggesting a significant role of IL-17A in orbital GPA compared to IIOD and orbital sarcoidosis. Thus, the presence of IL-17A is not only skewed in the serum of GPA patients (Abdulahad et al., 2008), but is largely expressed in orbital tissue as well. IL-17A is a pro-inflammatory cytokine produced by Th17, a distinct sub-set of T helper cells of Th1 T cells and Th2 T cells(Harrington et al., 2005).Therefore, this may indirectly imply an active role of Th17 in the inflammatory process in peripheral tissues of GPA, instead of Th1 T cells and Th2 T cells, which have shown conflicting findings in the past (Balding et al., 2001)(Csernok, 2003).During inflammation, IL-17Ainduces release of other inflammatory cytokines such as IL-6, GM-CSF, TGF- beta and TGF- alpha; attracting further immune cells such as neutrophils and lymphocytes to the target site.

IL-17A has also been suggested to have a role in the initiation and maintenance of granuloma formation. Indeed in sarcoidosis, expressions of IL-17A are reported to be increased in circulating memory T cells and in bronchus tissue granulomas (Ten Berge et al., 2012). In this study however, the involvement of IL-17A in granulomas induction and maintenance was not mirrored in our orbital GPA biopsies which had a higher IL-17A expression but with very few granuloma formations. Furthermore, the level of IL-17A was not significantly higher in our sarcoidosis sample. Hence, it appears that IL-17A may have a different mechanism in the pathogenesis of GPA compared to sarcoidosis. This may also account for the difference in the clinical presentation of the two diseases.

In our study there were no significant difference seen in the role of CD3, CD4, CD8 and CD134 in orbital GPA when compared to IIOD and orbital sarcoidosis. Although we did find that the level of CD3 and CD8 were higher in orbital GPA compared to IIOD, the odds ratio did not show any marked significance in the presence of these cells, unlike in the kidneys affected by GPA where CD8 cells were seen more than CD4 in affected tissues(Aasarød et al., 2001a).In addition, it was also interesting to observe that the CD4/CD8 ratio within the groups were all close to one and did not show any type pre-dominance. This seemed to imply that both CD4 and CD8 have equal roles in the inflammatory process in orbital GPA, IIOD and sarcoidosis. This is unexpected, especially with sarcoidosis, as CD4 T lymphocytes have been documented to be dominant in this disease (De Jager et al., 2008)where the CD4/CD8 ratio is usually more than 2.

CD134 in the orbital tissues in our study did not show a significant difference between the GPA and IOID, or GPA and sarcoidosis. CD134 was to be present in tissues affected by the GPA and is shown to be directly associated with disease activity. (Wilde et al.,

2009)Unfortunately, we did not investigate this association in GPA patients. Nonetheless, interestingly we were unable to find any reports linking CD134 with sarcoidosis or IIOD in previous studies, thus to our knowledge, this study may be the first to show such association.

5 Chapter 5: B Lymphocytes

5.1 B Cells in GPA

The role of B lymphocytes and memory plasma cells in immune regulation, antibody production and pathogenesis has been established in other auto-immune diseases such as systemic lupus erythematosus (Yoshida et al., 2010)(Dörner et al., 2009). Plasma B cells have been shown to be actively producing autoimmune antibodies in AAV, including ANCA (McQueen, 2012)(Jennette and Falk, 2008)(Fervenza, 2010).

Since the success of Rituximab i.e. a chimeric monoclonal antibody against the protein CD20 B cells, for the treatment of GPA, B cells have become implicated in the pathogenesis of GPA (Ferraro AJ 2008). Indeed, the presence of B cells in GPA tissue biopsies have been established, where aggregates of B cells in endonasal tissue affected with GPA were seen present in the vicinity of PR3+ cells (J Voswinkel, 2008). In addition, activated peripheral B cell levels have been linked to disease activity and severity in GPA (Popa et al., 1999).

In the lungs, granulomata obtained from the lower and upper respiratory tract affected by GPA is seen to contain follicle-like B cell clusters. It is then suggested that these granulomata could be the place of auto-antigen presentation, and formation of high-affinity ANCA could occur within these neo-formed ectopic or tertiary lymphoid-like tissue areas (Krämer et al., 2007).

In the kidneys, patients with refractory renal GPA were found to respond very well with Rituximab, again implying the role of B cells in the disease development in this organ (Selamet et al., 2007).However, in one study looking at renal tissues affected by GPA, glomeruli were noted to have an abundance of CD8 T cells and macrophages Nevertheless, surprisingly no B cells were detected in the tissues (Aasarød et al., 2001a).

Serum BAFF levels have also been reported to be elevated in patients with GPA and in nasal biopsies of patients affected with GPA, where BAFF has been shown to be present in these tissues (Zhao et al., 2012). Interestingly, BAFF and ANCA appear to function independently as shown in one study. BAFF levels were shown to be inversely correlated with ANCA titre indicating that BAFF does not drive ANCA production in GPA (Bader et al., 2010).

5.2 B cells in Sarcoidosis

B cells have been shown to play a part in the disease development of sarcoidosis as well. Large B-cells infiltrate the granulomatous tissue and increased molecular signs of antibody maturation were said to be indicative of direct involvement of B cells in local inflammatory processes in patients with sarcoidosis(Kamphuis et al., 2013). However, patients with severe chronic sarcoidosis were reported to have absolute B-cell lymphopenia and exhibited significantly decreased frequencies and total numbers of serum memory B cells. This is said to suggest that there is disturbed homeostasis, intrinsic signaling defects, and anergy within the peripheral B-cell compartments in severe chronic sarcoidosis (Lee et al., 2011).

Serum BAFF levels were also found to be significantly elevated in sarcoidosis patients when compared with healthy control patients, suggesting BAFF's role in the pathogenesis of this disease (Ueda-Hayakawa et al., 2013). Patients with active disease also had a higher BAFF level compared to inactive sarcoidosis patients. In addition, BAFF was shown to be strongly correlated to the serological markers for the disease, such as serum hypergammaglobulinemia and angiotensin converting enzyme levels (Saussine et al., 2012).

5.3 B Cells in IIOD

Reported investigations in the involvement of B cells in IIOD are scarce. In one study however, presence of B cells were mentioned in the histology of IIOD (McCarthy et al., 1993). Nevertheless, the effectiveness of Rituximab in the treatment of refractory IIOD have been shown, thus suggesting a positive influence of B cells in the pathogenesis of IIOD (Shao et al., 2013).

5.4 Objective

Rituximab appear to be very effective in the treatment of orbital GPA, sarcoidosis, as well as IIOD, indicating a role of B cells in all three diseases. However, the extent of involvement of this cell in each disease is unclear. We wanted to discover if there exist a difference in the B cell presence between orbital biopsies of GPA, sarcoidosis and IIOD, which may aid in differentiating them apart, histologically. In addition, we also wanted to look at the involvement of BAFF and APRIL in these tissues.

Our specific objectives in this part of the study are:

1. to demonstrate the occurrence of CD20, BAFF, APRIL, BAFF-R, TACI and BCMA in orbital tissue biopsies of GPA, sarcoidosis and IIOD,
2. to compare the occurrences of CD20, BAFF, APRIL, BAFF-R, TACI and BCMA between orbital tissue biopsies of GPA and IIOD, and
3. to compare the occurrences of CD20, BAFF, APRIL, BAFF-R, TACI and BCMA between orbital tissue biopsies of GPA and orbital sarcoidosis.

5.5 Method

5.5.1 Patient and Tissue Selection

The same paraffin blocks from the previously selected 25 orbital GPA blocks, 25 IIOD blocks and 13 orbital sarcoidosis blocks were used.

5.5.2 Antibodies

IHC was performed for cell markers CD20cy (Clone L26; M0755, DAKO, Cambridgeshire, UK), cytokine BAFF (TNFSF13B Antibody, NBP1-86933, Novus Biologicals, Cambridge, UK) and BAFF-R (11C1; ab16232Abcam, Cambridge, UK), IL-17 (IL-17A; AHP455G AbDSerotec, Kidlington, UK).

We were unable to obtain a suitable APRIL, TACI and BCMA antibody within the time frame of this study.

5.5.3 Slide Preparations

Slide preparations and stainings for each antibody were performed on the same day and under the same laboratory conditions for all cases. Tissue sections from the blocks were cut using a sledge microtome at a thickness of 5 micrometer and mounted on Superfrost-plus object glass slides. The slides were left on a hot plate at 40°C for 60 minutes and then incubated overnight at 37°C before use.

5.5.4 Immunohistochemistry Staining of Fixed Paraffin Tissues (Table 5.1)

Tissue sections were de-waxed in two changes of xylene (each 5 to 10 minutes) with agitation at regular intervals, then twice with 100% ethanol (10 to 20 seconds) and then hydrated in 90%, 70%, and 50% ethanol, each for 10 to 20 seconds, followed by two washes in water. Sections were then incubated in the appropriate antigen retrieval solution where Tris/EDTA pH 9.0 (H-3301, Vector Labs, UK) was used for CD20, and sodium citrate pH 6.0 (H-3300, Vector Labs, UK) for BAFF and BAFF-R as recommended by the supplier. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes.

5.5.5 Automated Staining for CD20

CD20cy using concentration of 1:200; as per manufacturer recommendations, were performed by the DAKO Autostainer Plus. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Positive and negative controls with tonsil tissue were also performed. (Table 0.1)

5.5.6 Manual Staining for BAFF and BAFF-R

5.5.6.1 Manual Staining for BAFF

For BAFF, after de-waxing and antigen retrieval as previously described above, slides were removed from the staining rack, placed in a moist chamber and tissues were kept

hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). BAFF primary antibody, diluted in blocking solution at concentrations of 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

5.5.6.2 Manual Staining for BAFF-R

Primary antibody for BAFF-R were titrated using inflamed tonsil tissues until an optimal tissue stain were achieved.

After de-waxing and antigen retrieval as previously described above, slides were removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). BAFF-Rprimary antibodies at a concentration of 1:50, as per supplier recommendation, diluted in blocking solution were added and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit K5001 HRP/DAB+, Rabbit/Mouse; DAKO, UK were used for visualization. Finally, the slides were counter-stained with haematoxylin, then mounted with DPX mounting medium and cover slips, and allowed to dry overnight before imaging. Positive and negative slides with tonsil tissue for each antibody were also performed.

5.6 Imaging and Image Analysis

5.6.1 IHC

ADCIS ® Stereology Toolkit frame count programme was used as before. Patient's identity and the diagnosis for each slide were masked during the counting process. For IHC, a total of 50 count frames (instead of 20) were randomly generated by the software for all slide images making up a total area of 50 mm^2 analysed for every slide. As previously done, only stained cells within the frame and not touching the exclusion lines were included in the count, and the software then generated the total number of marked cells to generate the total count.

5.6.2 Validation process for cell and tissue counts

Identification of positive stained cells were counter checked and validated by a senior pathologist (secondary supervisor) who was also masked from patients' identities and diagnoses. A portion of the slides were also re-counted to ensure total counts were reproducible.

5.7 Data analysis

Cell counts were entered into an Excel spreadsheet. The Mann Whitney test was performed with SPSS17 for quantitative comparisons of the individual stained cells between GPA and IIOD, and GPA and orbital sarcoidosis. Odds ratio was calculated via the 2x2 frequency table.

5.8 Results

5.8.1 T Cell Identification in Orbital GPA, IIOD and Sarcoidosis

CD20 and BAFF-R were all present in orbital GPA tissue biopsies as well as in tissue biopsies of sarcoidosis and IIOD.

We were unsuccessful in getting a convincing stain for BAFF in our control samples despite using various antibody concentrations. (Table 5.1) Attempts on staining BAFF on diseased tissue i.e. GPA, also did not successfully produce clear and convincing results. (Figure 5.3)

Table 5.1: Immunohistochemistry protocol for B cells investigation

Cell type	Antigen retrieval	Epitope retrieval	Mode of staining	Antibody concentration	Incubation time (temperature)	Result
CD20	Tries/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Autotimer	1:400	_	Positive
BAFF-R	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Positive
BAFF	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:10	24 hours (4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:100	24 hours (4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours (4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:400	24 hours (4^{o}C)	Unsuccessful

5.8.2 Cell Count Comparison between GPA and IOID

Count comparison for CD20 and BAFF-R between GPA and IIOD are summarised in Table 5.2. CD20 counts were found to be similar between GPA and IIOD (p=0.2). However, BAFF-R levels were found to be significantly higher in orbital GPA compared to IIOD (p=0.01). Further odds ratio calculation showed that a high counts of BAFF-R found in an orbital biopsy was 7 times more likely to be from GPA tissues than from IIOD (OR= 6.6). (Table 5.2) (Figure 5.1&Figure 5.2)

Table 5.2: Quantitative CD20 and BAFF-R comparisons between orbital GPA and IIOD (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA	IOID	p	OR	CI
CD20 (mean+/-SEM)	1025+/-232	1026+/-363	0.2		
BAFF-R (mean+/-SEM)	486+103 (12635)	203+/-34	0.01*	6.6*	0.64 – 55.66

* = significant odds ratio

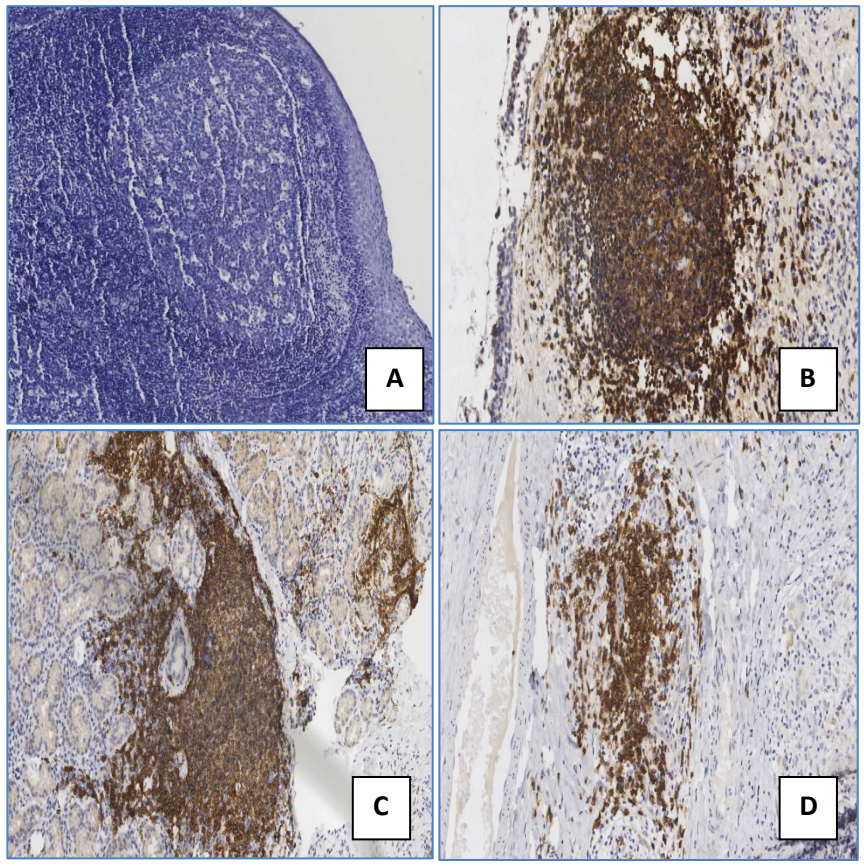
5.8.3 **Cell Count Comparison between GPA and Sarcoidosis**

Count comparison for CD20 and BAFF-R between GPA and sarcoidosis are summarised in Table 0.3. CD20 counts were found to be similar between GPA and orbital sarcoidosis (p=0.2). However, BAFF-R expressions were found to be significantly higher in orbital GPA compared to orbital sarcoidosis (p=0.02). Further odds ratio calculation showed that a high BAFF-R count in an orbital biopsy was 6 times more likely to be from GPA tissues than from orbital sarcoidosis (OR= 6). (Table 5.3) (Figure 5.1&Figure 5.2)

Table 5.3: Quantitative CD20 and BAFF-R counts and comparisons between orbital GPA and orbital sarcoidosis (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA	Orbital sarcoidosis	p	OR	CI
CD20 (mean+/-SEM)	1025+/-232	932+/-213	0.4		
BAFF-R (mean+/-SEM)	486+103	238+/-135	0.02*	6*	0.65 – 55.66

* = significant odds ratio



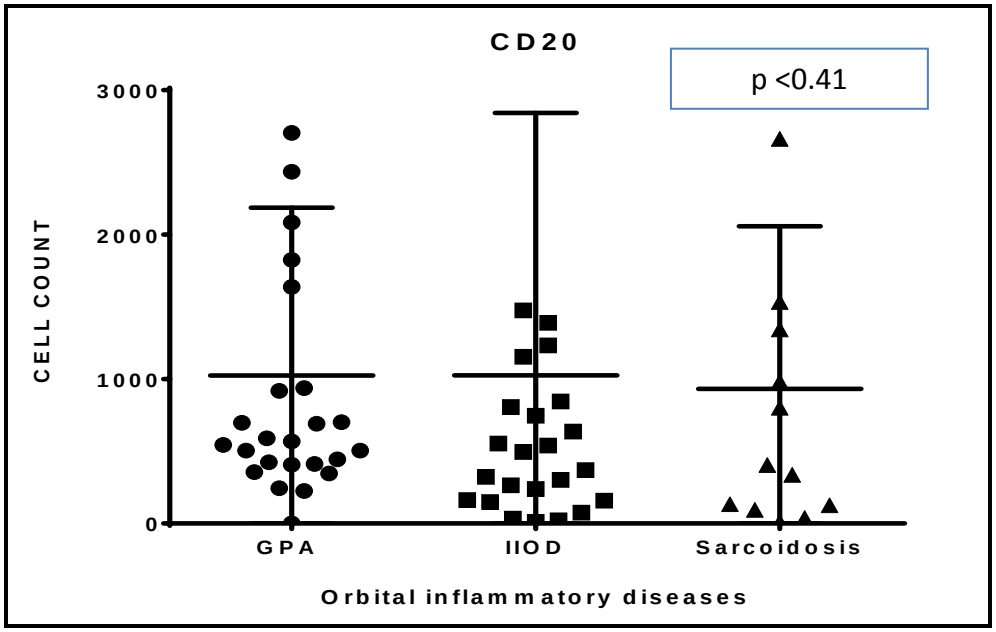
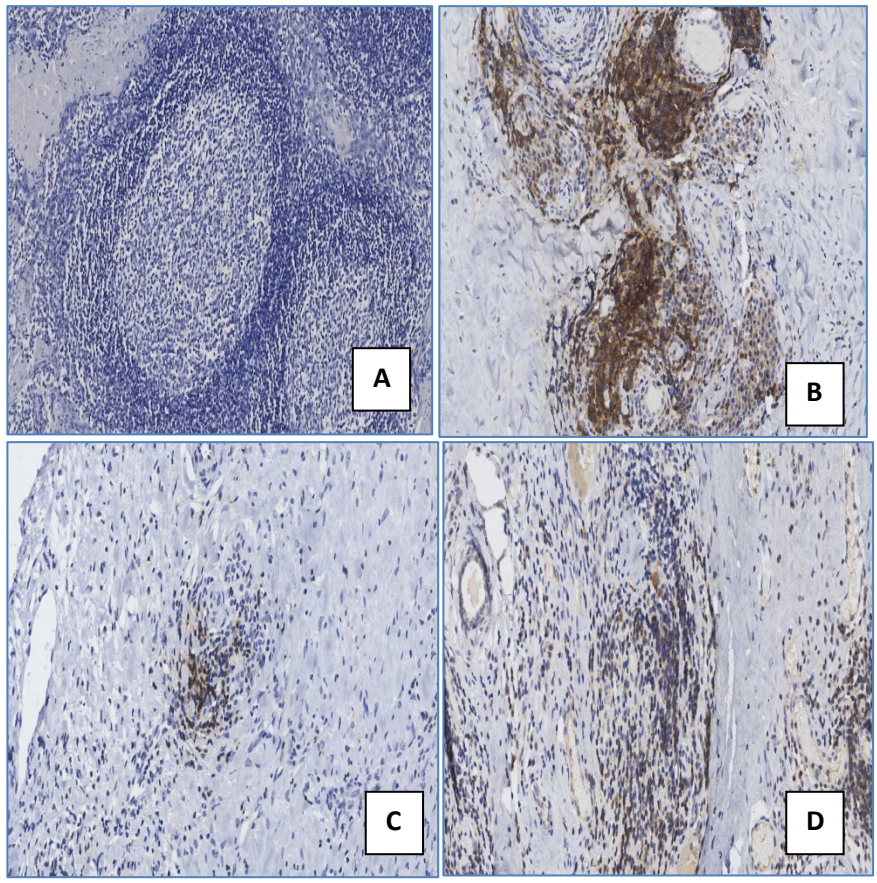


Figure 5.1: CD20 tissue stainings with HRP/DAB+. A: Negative CD20 staining on an inflammed tonsil (primary antibody not added, x100), B: CD20 staining on a GPA orbital tissue. (HRP/DAB+, Rabbit/Mouse; x40), C: CD20 staining on an IIOD tissue (HRP/DAB+, Rabbit/Mouse; x40), D: CD20 staining on a or orbital sarcoidosis tissue (HRP/DAB+, Rabbit/Mouse; x40), E: Scatter plot of CD20 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing no significant difference between them (p<0.41).



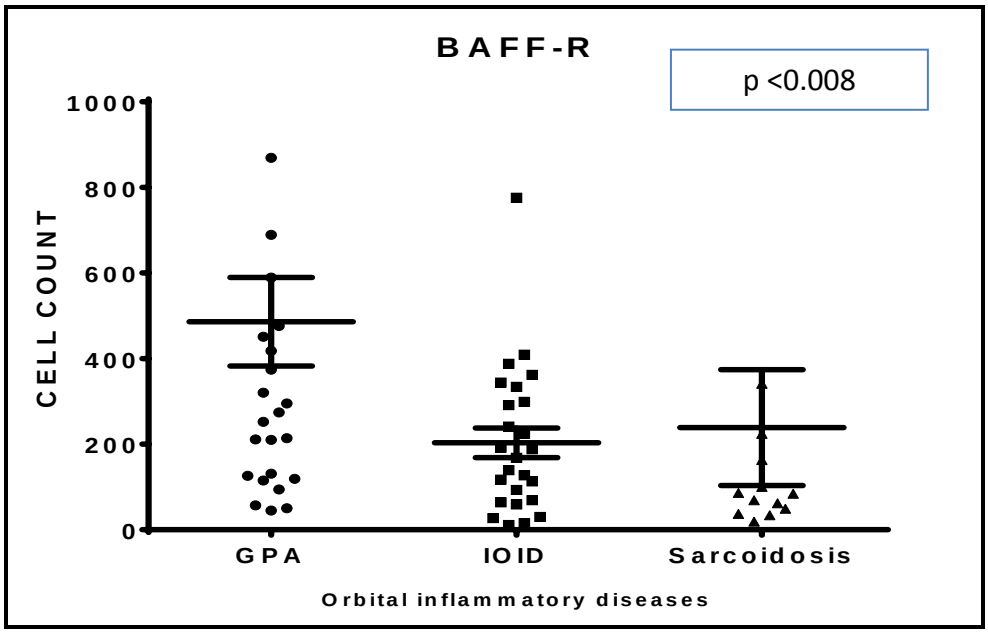


Figure 5.2: BAFF_R tissue stainings with HRP/DAB+. A: Negative BAFF-R staining on an inflammed tonsil (primary antibody not added, x100), B: BAFF-R staining on a GPA orbital tissue (HRP/DAB+, Rabbit/Mouse; x100), C: BAFF-R staining on an IIOD (HRP/DAB+, Rabbit/Mouse; x100), D: BAFF-R staining on an orbital sarcoidosis tissue. (HRP/DAB+, Rabbit/Mouse; x100), E: Scatter plot of BAFF-R showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing significant difference between them (p<0.001). Dunn's multiple comparisons revealing BAFF-R significantly more in GPA compared to both IOID and orbital sarcoidosis.

5.9 Discussion

In this section of the study, we were able to demonstrate the presence of CD20 and BAFF-R in orbital GPA, sarcoidosis and IIOD tissues.

The presence of B cells in our study corresponds with other studies that show the involvement of B cells in GPA. B cells not only have been documented to have a role in the pathogenesis of GPA, but have also been reported to have an influence in disease activity and severity in GPA (Popa et al., 1999)(Eriksson et al., 2010). In addition, Rituximab, a monoclonal anti-B cell CD20 antibody, has recently been found to be very effective in treating refractory AAV (Jones et al., 2010)(Khan et al., 2010)(Onal et al., 2008a), including refractory ophthalmic GPA (Joshi et al., 2011)(Hinze and Colbert, 2008). Thus this further confirms the association of this immune cell in the disease process of GPA.

We did not find a significant difference in the presence of CD20 in GPA when compared to IIOD and orbital sarcoidosis. This indicates that B cells may have equal significance in the disease process in all three orbital inflammatory diseases. We were unfortunately unable to establish the presence of B cell activating factor (BAFF) in our tissue biopsies despite following tested published protocols and varying the antibody concentrations. The failure in staining BAFF in our sample tissues could be due to two possibilities: (1) our tissue samples were archived tissues and not fresh tissues, hence it may be possible that the cytokine may have not been preserved thus preventing good staining, 2) the paraffin preparation may have degraded the fragile BAFF cytokines in our sample tissues.

We were however successful in staining BAFF-R in all of our sample tissues. In one study, it is reported that the critical functions of BAFF in B and T cell biology are highly influenced by the expression of BAFF receptors on immune cells (Ng et al., 2004). BAFF-R specifically interacts with BAFF (Thompson et al., 2001), and has a higher affinity to this cytokine compared to TACI and BCMA. Thus, the positive presence of BAFF-R in these sample tissues indirectly suggests a positive BAFF activity in orbital GPA compared to IIOD and sarcoidosis.

Although CD20 counts were found similar in all three diseases, our orbital GPA tissues were found to exhibit more BAFF-R compared to the other two diseases. Indirectly, this may also imply that the activity of BAFF may be higher in orbital GPA as well, compared to sarcoidosis. B cells indeed have been shown to be involved in the pathogenesis of auto-immune diseases, including GPA. In addition, plasma B cells have also been reported to produce auto-immune antibodies including ANCA, which is linked to the pathogenesis of GPA. Thus, although the number of B cells may be similar in all three diseases, the significantly higher levels of BAFF-R found in orbital GPA suggests that the activity of B cells in this disease differs to that of sarcoidosis and IIOD.

The marked expressions of BAFF-R seen in orbital GPA indicate a potentially higher BAFF level in this disease. Therefore, in GPA, although the number of B cells present in peripheral tissues may be similar to sarcoidosis and IIOD, these active B cells may survive longer and may have a more prolonged and sustained activity under the influence of BAFF. This may explain the difference in the manifestations and severity of orbital GPA compared to IIOD and orbital sarcoidosis.

The presence of B cells in orbital GPA as well as sarcoidosis and IIOD also affirms the potential effectiveness of Rituximab treatment, which targets these cells in these diseases.

5.10 B- cell staining trials

5.10.1 Trails of BAFF staining at different primary antibody concentrations

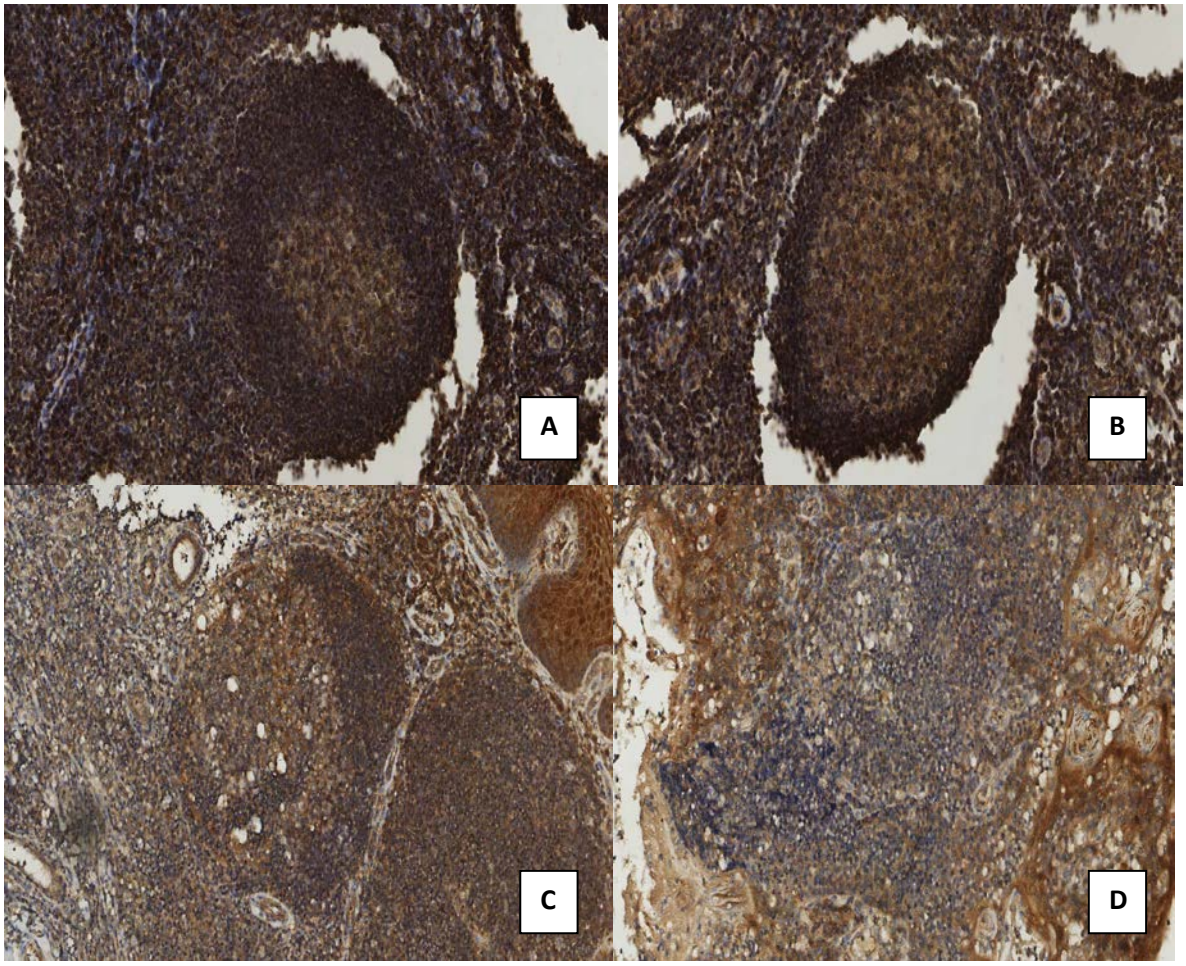


Figure 5.3: Unsuccessful BAFF staining on an inflamed tonsil tissues at different concentrations with HIER sodium citrate pH6 epitome retrieval and HRP/BAD+. A: Unsuccessful BAFF staining (1:10 concentration) (HRP/DAB+, Rabbit/Mouse; x40), B: Unsuccessful BAFF staining (1:50 concentration). (HRP/DAB+, Rabbit/Mouse; x40), C: Unsuccessful BAFF staining (1:100 concentrations). (HRP/DAB+, Rabbit/Mouse; x40), D: Unsuccessful BAFF staining (1:200 concentration). (HRP/DAB+, Rabbit/Mouse; x40)

6 Chapter 6: Macrophages

6.1 Macrophages in GPA

The presence of CD68 has been demonstrated in GPA tissues (Rasmussen et al., 1990). Macrophages in GPA tissue biopsies have been observed to be loaded with tissue debris(Mackiewicz et al., 2005), and in the lungs, multinucleated giant cells are shown to express osteoclastic-like enzymes, said to possibly cause tissue destruction(Park et al., 2012). In the kidneys affected by GPA, significant correlation was found for the glomerular infiltration of CD68-positive macrophages with the presence of glomerular necrosis, as well as with the number of glomeruli with crescents. In addition, a significant correlation was also found for the interstitial, as well as for the glomerular infiltration of CD68-positive macrophages with serum creatinine concentration at the time of biopsy (Weidner et al., 2004).

6.2 Macrophages in Sarcoidosis and IIOD

As with GPA, macrophage phenotypes in sarcoidosis and IIOD have also not been extensively reported. There are almost no reports describing the involvement of different macrophage subtypes in the pathogenesis of IIOD. In sarcoidosis however, presence of CD68 in sarcoidosis have been reported and is shown to be able to help in histological diagnosis of sarcoidosis on small transbronchial biopsies (Menestrina et al., 1992)(Jaskiewicz et al., 2006). In one study, CD163 was demonstrated to be expressed in inflammed lungs in sarcoidosis despite regarded as an M2 macrophage marker (Abdullah et al., 2012). IL-23 polymorphism has also have been associated with sarcoidosis (Judson et al., 2012)especially in sarcoid uveitis(Kim et al., 2011).The defect in the TLR9 pathway

is said to be responsible in the production of cytokine IL-23 in sarcoidosis(Veltkamp et al., 2010).

6.3 Objective

Macrophages are the predominant cellular component in granulomatous inflammation which is one the main feature in the histology and pathology of GPA as well as sarcoidosis. However, very little data on macrophage phenotypes, either during the course of disease or in different organs in these diseases, exist. We therefore wanted to further investigate the role of macrophages in orbital GPA.

The main of objective for this part of the study is:

1. To demonstrate the occurrence of CD68, CD163, CD204, macrophages and cytokine AIF1 and IL-23 in orbital tissue biopsies of GPA, sarcoidosis and IIOD
2. To compare the occurrences of CD68, CD163, CD204 macrophages and cytokine AIF1 and IL-23 between orbital tissue biopsies of GPA and IIOD
3. To compare the occurrences of CD68, CD163, CD204 macrophages and cytokine AIF1 and IL-23 between orbital tissue biopsies of GPA and orbital sarcoidosis

6.4 Method

6.4.1 Tissue selection

The same paraffin blocks from the previous selection of 25 patients from the orbital GPA group, 25 patients from the IIOD group and 13 paraffin of orbital sarcoidosis were used.

6.4.2 Antibodies

IHC was performed for cell markers CD68 (Clone PG-M1; M0876, DAKO, Cambridgeshire, UK), CD163 (5C6-FAT, BM4041, Novus Biologicals, Cambridge, UK) CD204 (Macrophage Scavenger Receptor Antibody, NBP1-88125, Novus Biologicals, Cambridge, UK), AIF 1 (NB100-94240, Novus Biologicals, Cambridge, UK) and IL-23 (H-113; sc-50303, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA).

6.4.3 Slide preparation

Slide preparations and stainings for each antibody were performed on the same day and under the same laboratory conditions for all cases. Tissue sections from the blocks were cut using a sledge microtome at a thickness of 5 micrometers and mounted on Superfrost-plus object glass slides. The slides were left on a hot plate at 40°C for 60 minutes and then incubated overnight at 37°C before use.

6.4.4 Immunohistochemistry staining of fixed paraffin tissues (Table 6.1, Table 6.2,Table 6.3)

Tissue sections were de-waxed in two changes of xylene (each 5 to 10 minutes) with agitation at regular intervals, then twice with 100% ethanol (10 to 20 seconds) and then hydrated in 90%, 70%, and 50% ethanol, each for 10 to 20 seconds, followed by two washes in water. Sections were then incubated in the appropriate antigen retrieval solution and primary antibodies. Staining protocols that were attempted for each antibody are described below.

6.4.5 Automated staining for CD68 (Table 6.1)

For CD68, Tris/EDTA pH 9.0 (H-3301, Vector Labs, UK) was used for antigen retrieval. Thereafter, heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes. IHC staining for CD68 (1:400) was then performed by the DAKO Autostainer Plus. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, DAKO, UK) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging. Positive and negative slides with tonsil tissue for each antibody were also performed.

6.4.6 **Manual staining for IL-23 staining (Table 6.1)**

After de-waxing, sodium citrate pH 6.0 (H-3300, Vector Labs, UK) was used for antigen retrieval. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes. Slides were then removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). IL-23 (1:800), diluted in blocking solution was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit K5001 HRP/DAB+, DAKO, UK were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging. Positive and negative slides with tonsil tissue for each antibody were also performed.

6.4.7 **Manual staining forCD163 on tonsil (control tissue) (Table 6.1)**

6.4.7.1 Trial with variation in Protein K Antigen Retrieval timing

After de-waxing, slides were removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissue using a hydrophobic barrier pen (ImmEdge Pen H-4000). Proteinase K was used for antigen retrieval; placed on top of the tissue (within the barrier ring) and was left for various timings (3 minutes (recommended time by manufacturer), 5 minutes and 10

minutes), to obtain the best staining. CD163 primary antibody, diluted in blocking solution at 1:50 concentration, as recommended by supplier, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit K5001 HRP/DAB+, Rabbit/Mouse were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

6.4.7.2 Trial variation with differing antibody concentrations (antigen retrieval with Protein K for 3 minutes applied)

After de-waxing, slides were removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissue using a hydrophobic barrier pen (ImmEdge Pen H-4000). Proteinase K was used for antigen retrieval; placed on top of the tissue (within the barrier ring) and was left for 3 minutes (as per-supplier protocol). CD163 primary antibody, diluted in blocking solution at concentrations of 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

6.4.8 **Manual staining for AIF1 on tonsil (control tissue) (Table 6.2)**

6.4.8.1 Trial with Sodium citrate pH 6.0 antigen retrieval and variation of primary antibody concentration

After de-waxing, sodium citrate pH 6.0 (H-3300, Vector Labs, UK) was used for antigen retrieval. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes. Slides were then removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). AIF1 primary antibody, diluted in blocking solution at concentrations of 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, DAKO, UK) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging. Positive and negative slides with tonsil tissue for each antibody were also performed.

6.4.8.2 Trial with Tris/EDTA pH9.0 antigen retrieval and variation of primary antibody concentration

After de-waxing, Tris/EDTA pH9.0 (H-3301, Vector Labs, UK) was used for antigen retrieval. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes. Slides

were then removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). CD204 primary antibody, diluted in blocking solution at concentrations of 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

6.4.8.3 Trial with Proteinase K antigen retrieval

After de-waxing, slides were removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissue using a hydrophobic barrier pen (ImmEdge Pen H-4000). Proteinase K was used for antigen retrieval; placed on top of the tissue (within the barrier ring) and was left for various timings (3 minutes (recommended time by manufacturer), 5 minutes and 10 minutes), to obtain the best staining. CD163 primary antibody, diluted in blocking solution at 1:50 concentration (as recommended by supplier), was added and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

6.4.9 **Manual CD204 staining on tonsil (control tissue) (Table 6.3)**

After de-waxing, sodium citrate pH 6.0 (H-3300, Vector Labs, UK) was used for antigen retrieval. Heat induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X, Code S3006; DAKO UK) for 5 minutes. Slides were then removed from the staining rack, placed in a moist chamber and tissues were kept hydrated with Tris-buffer solution. A ring was then made around the tissues for all slides using a hydrophobic barrier pen (ImmEdge Pen H-4000). CD204 primary antibody, diluted in blocking solution at concentrations of 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400, was added, and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+ and Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse) were used for visualization. Finally, the slides were counterstained with haematoxylin, then mounted with DPX mounting medium and cover slips and allowed to dry overnight before imaging.

Table 6.1: Immunohistochemistry protocol for macrophages investigation (CD68, IL-23 and CD163)

Cell type	Antigen retrieval	Epitope retrieval	Mode of staining	Antibody conc.	Incubation time	Result
CD68	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Autostainer	1:400	_	Positive
IL-23	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:800	24hours (4^{o}C)	Positive
CD163	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (5 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Unsuccessful
	ProteinaseK (10 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:10	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:20	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:100	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours (4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:400	24 hours (4^{o}C)	Unsuccessful

Table 6.2: Immunohistochemistry protocol for macrophages investigation (AIF1)

Cell type	Antigen retrieval	Epitope retrieval	Mode of staining	Antibody concentration	Incubation time	Result
AIF1	sodium citrate pH 6.0	95^{o}Heat pressure cooker)	Manual	1:10	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:20	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:100	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:400	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:10	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:20	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:100	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours(4^{o}C)	Unsuccessful
	Tris/EDTA pH 9.0	95^{o}Heat (pressure cooker)	Manual	1:400	24 hours(4^{o}C)	Unsuccessful
	Proteinase K (3 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful
	Proteinase K (5 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful
	ProteinaseK(10 min)	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful

Table 6.3: Immunohistochemistry protocol for macrophages investigation (CD204)

Cell type	Antigen retrieval	Epitope retrieval	Mode of staining	Antibody concentration	Incubation time	Result
CD204	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:10	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:20	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:50	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:100	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:200	24 hours(4^{o}C)	Unsuccessful
	sodium citrate pH 6.0	95^{o}Heat (pressure cooker)	Manual	1:400	24 hours(4^{o}C)	Unsuccessful

6.5 Imaging and image analysis

6.5.1 IHC

ADCIS ® Stereology Toolkit frame count programme was used as before. Patient's identity and the diagnosis for each slide were masked during the counting process. For IHC, a total of 50 count frames (instead of 20) were randomly generated by the software for all slide images making up a total area of 50mm^2 analysed for every slide. As previously described, only stained cells within the frame and not touching the exclusion lines were included in the count, and the software then generated the total number of marked cells to generate the total count.

6.5.2 Validation process for cell and tissue counts

Identification of positive stained cells were counter checked and validated by a senior pathologist (secondary supervisor) who was also masked from patients' identities and diagnoses. A portion of the slides were also re-counted to ensure total counts were reproducible.

6.5.3 Data analysis

Cell counts were entered into an Excel spreadsheet. The Mann Whitney test was performed with SPSS17 for quantitative comparisons of the individual stained cells between GPA and IIOD, and GPA and orbital sarcoidosis. Odds ratio was calculated via the 2x2 frequency table.

6.6 Results

6.6.1 Macrophage identification in orbital GPA, IIOD and sarcoidosis

CD68 and IL-23 postive stained cells were found present in all orbital GPA tissue biopsies as well as in tissue biopsies of sarcoidosis and IIOD.

We were unsuccessful in getting convincing stains for CD163, CD204 and cytokine AIF1 in our control samples despite using various antibody concentrations (Figure 6.7 - Figure 6.12).

6.6.2 Macrophage comparison between GPA and IOID

Count comparison for CD68 and IL-23 between GPA and IIOD are summarised in Table 6.4. CD68 counts as well as IL-23 were markedly higher in GPA compared to IIOD where p values were <0.001 for both diseases. Further odds ratio calculation showed that high levels of CD68 in tissue biopsy were more likely to be from GPA and not for IIOD. As for IL-23, odds ratio calculations showed that high levels of IL-23 in tissue biopsy were nine time more likely to be from GPA than IIOD in an orbital biopsy were 7 times more likely to be from GPA tissues than from IIOD (OR= 9.3). (Table 6.4) (Figure 6.1&Figure 6.2)

Table 6.4: Quantitative CD68 macrophage and IL-23 cytokines cell counts and comparisons between orbital GPA and IIOD (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA	IOID	p	OR	CI
CD68 (mean+/-SEM)	2202+/-216	1152+/-152	<0.001*	∞*	∞
IL-23 (mean+/-SEM)	3186+/-338	1461+/-208	<0.001*	9.3*	1.05 – 82.78

* = significant odds ratio (p<0.05)

6.6.3 Macrophage comparison between GPA and sarcoidosis

The count comparison for CD68 and IL-23 between GPA and orbital sarcoidosis is summarised in Table 6.5. CD68 counts were similar between GPA and orbital sarcoidosis (p=0.1). Nevertheless, IL-23 was found to be significantly higher in orbital GPA compared to orbital sarcoidosis (p=0.02)(Figure 5.3). Further odds ratio calculation showed that high levels of IL-23 in an orbital biopsy were more likely to be from GPA tissues and not from orbital sarcoidosis. (Table 6.5)(Figure 6.1&Figure 6.2)

Table 6.5: Quantitative CD68 macrophage and IL-23 cytokines cell counts and comparisons between orbital GPA and sarcoidosis (cell count performed in 50x1mm^2 (50mm^2) field in each case)

Cells	Orbital GPA	Orbital sarcoidosis	p	OR	CI
CD68 (mean+/-SEM)	2202+/-216	1561+/-300	0.1		
IL-23 (mean+/-SEM)	3186+/-338	1461+/-326	0.02*	∞*	∞

* = significant odds ratio ($p < 0.05$)

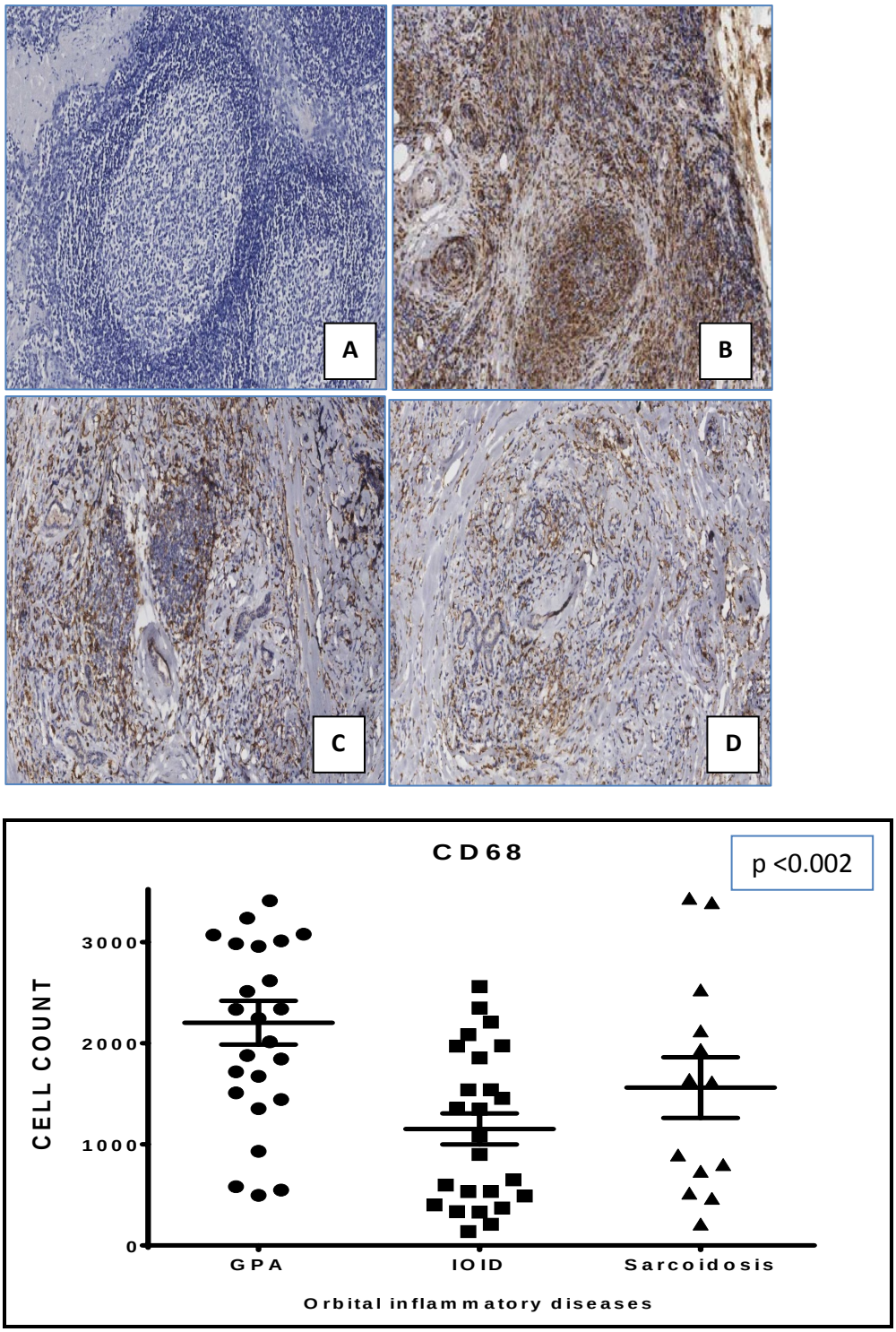


Figure 6.1: CD68 tissue stainings with HIER Tris/EDTA pH9 antigen retrieval and HRP/DAB+. A: Negative control slide on an inflammed tonsil (CD68 primary antibody not added, x100), B: CD68 staining on a GPA orbital tissue (HRP/DAB+, Rabbit/Mouse; x100), C: CD68 staining on an IIOD (HRP/DAB+, Rabbit/Mouse; x100), D: CD68 staining on orbital tissues from patients with idiopathic orbital inflammatory disease. (HRP/DAB+, Rabbit/Mouse; x100), E: Scatter plot of CD68 showing range of counts and standard error of mean bar for each of the three orbital diseases, with ANOVA test showing significant difference between them (p<0.03). Dunn's multiple comparison revealing main difference between and GPA and IOID.

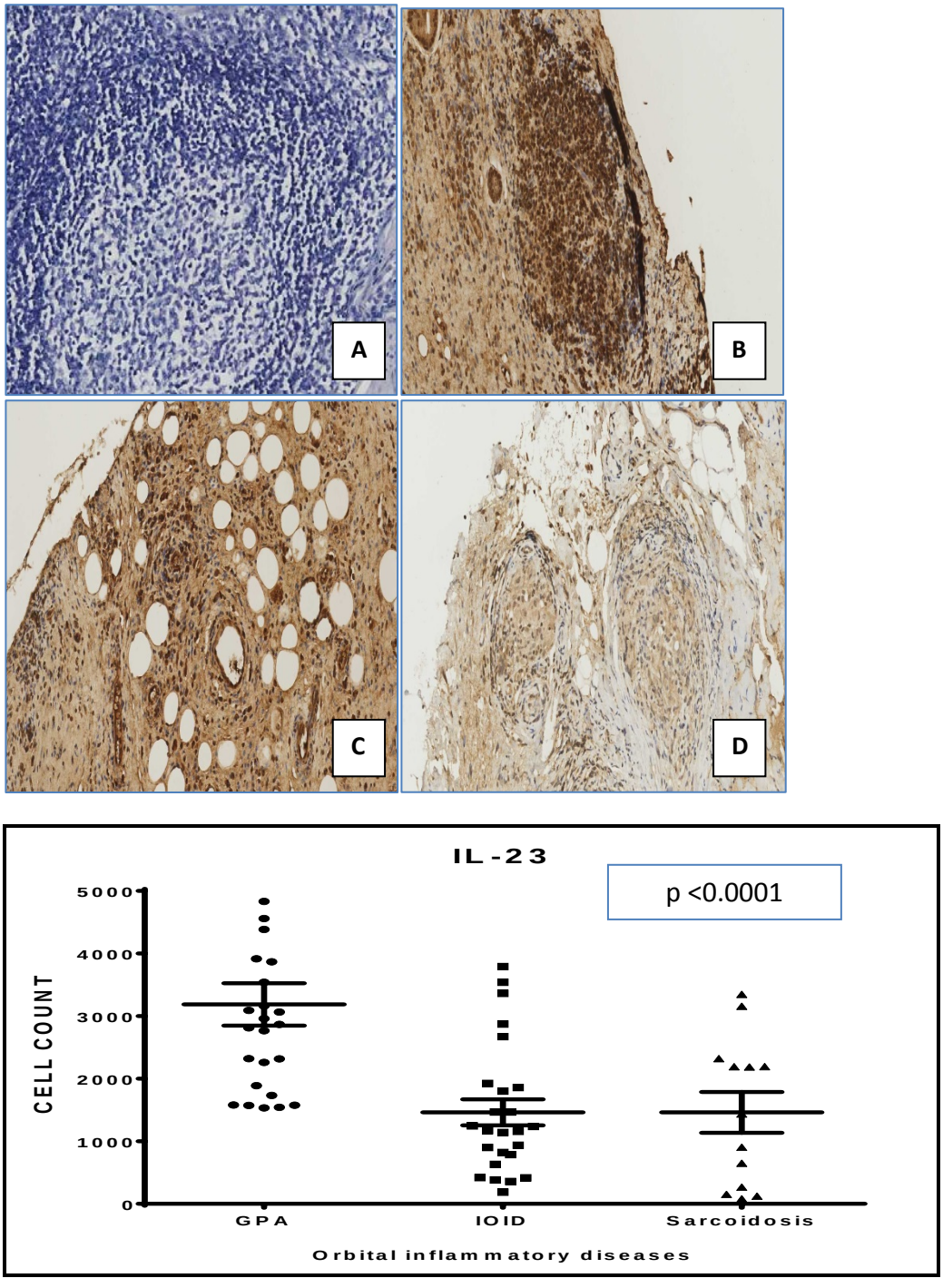


Figure 6.2: IL-23 tissue stainings with HIER with sodium citrate pH6 antigen retrieval and HRP/DB+. A: Negative control slide on an inflammed tonsil (IL-23 primary antibody not added, x100), B: IL-23 staining on a GPA orbital tissue (HRP/DAB+, Rabbit/Mouse; x100), C: IL-23 staining on an IIOD (HRP/DAB+, Rabbit/Mouse; x100), D: IL-23 staining on an orbital sarcoidosis tissue. (HRP/DAB+, Rabbit/Mouse; x100), E: Scatter plot of IL-23 showing range of counts and standard error of mean bar for each of the three orbital diseases with ANOVA test showing significant difference between them (p<0.001). Dunn's multiple comparisons revealing IL-23 significantly more in GPA compared to both IOID and orbital sarcoidosis.

6.6.4 **IL-17A and IL-23 correlation**

Based on our IL-17A and IL-23 results, we further analysed the possible correlation between IL-17A and IL-23. All 3 groups failed to show any significant correlation between the 2 entities (orbital GPA p=0.9; IOID p=0.5; orbital sarcoidosis p=0.2. (Figure 6.3 - Figure 6.5) However, in the orbital GPA group, we observed 2 outliers in our data. Upon removal of these data, there appear to be a possible positive significant correlation (Pearson r = 0.43, p=0.02) between IL-17A and IL-23, and linear regression revealed that for every increase of IL-23 by 8 counts there is a 100 count increase of IL-17A (p=0.04). (Figure 6.6)

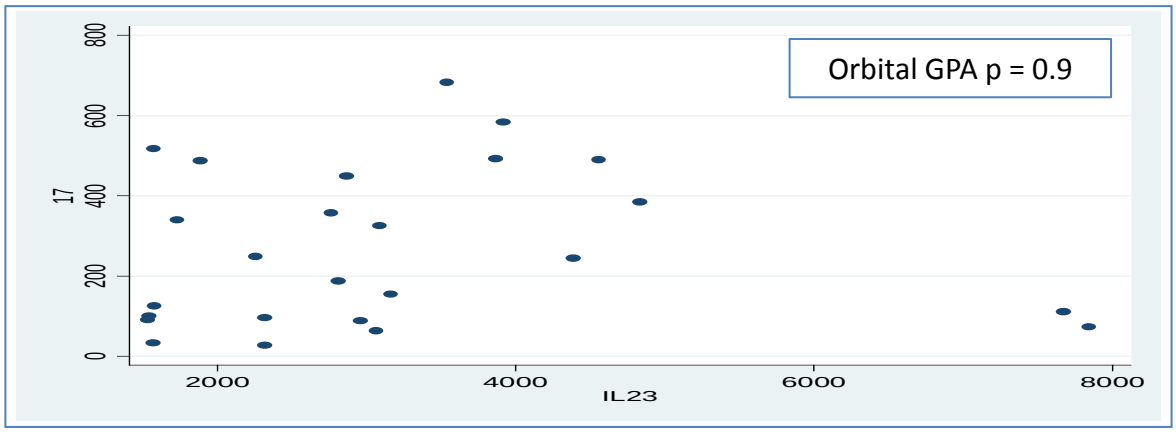


Figure 6.3: Scatter plot for correlation analysis between IL-17 and IL-23 in GPA. Results show no correlation between IL-17 and IL-23 in GPA

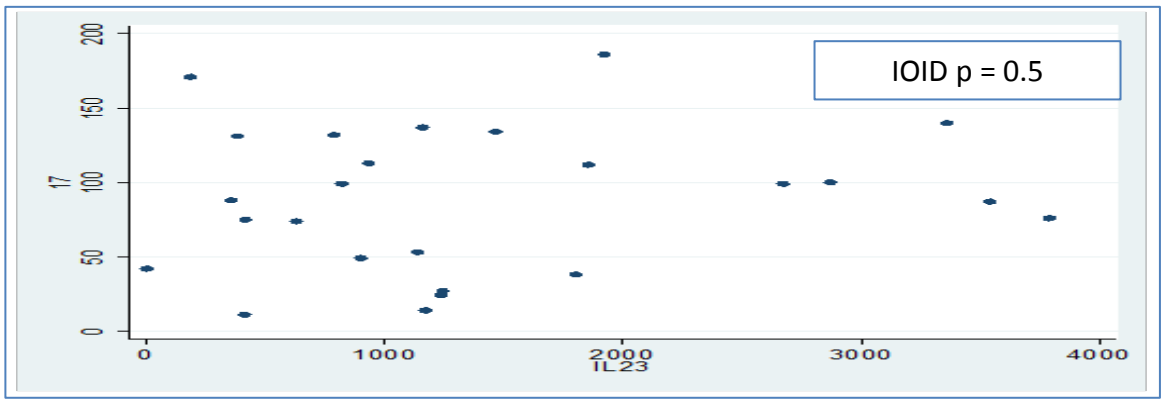


Figure 6.4: Scatter plot for correlation analysis between IL-17A and IL-23 in IIOD. Results show no correlation between IL-17A and IL-23 in IIOD.

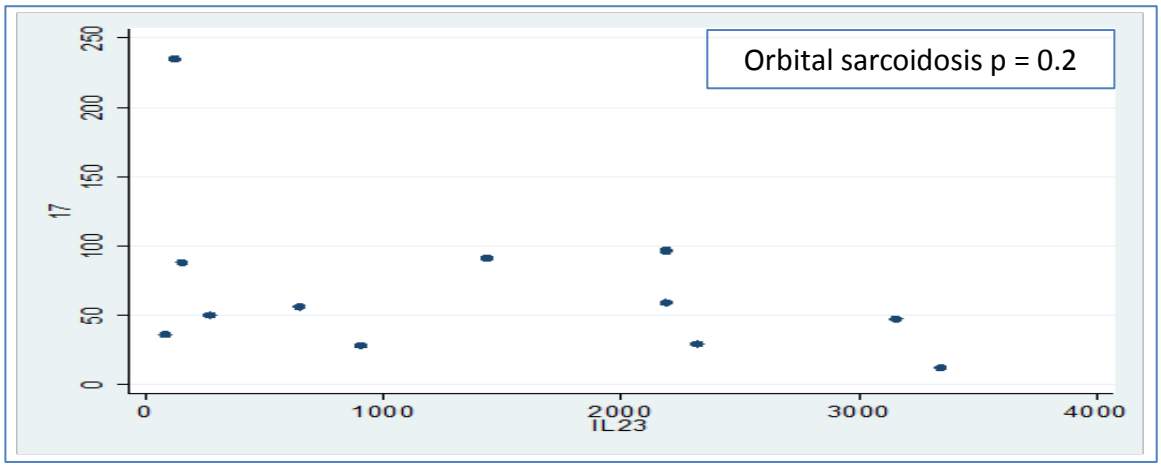


Figure 6.5: Scatter plot for correlation analysis between IL-17A and IL-23 in sarcoidosis. Results show no correlation between IL-17A and IL-23 in sarcoidosis.

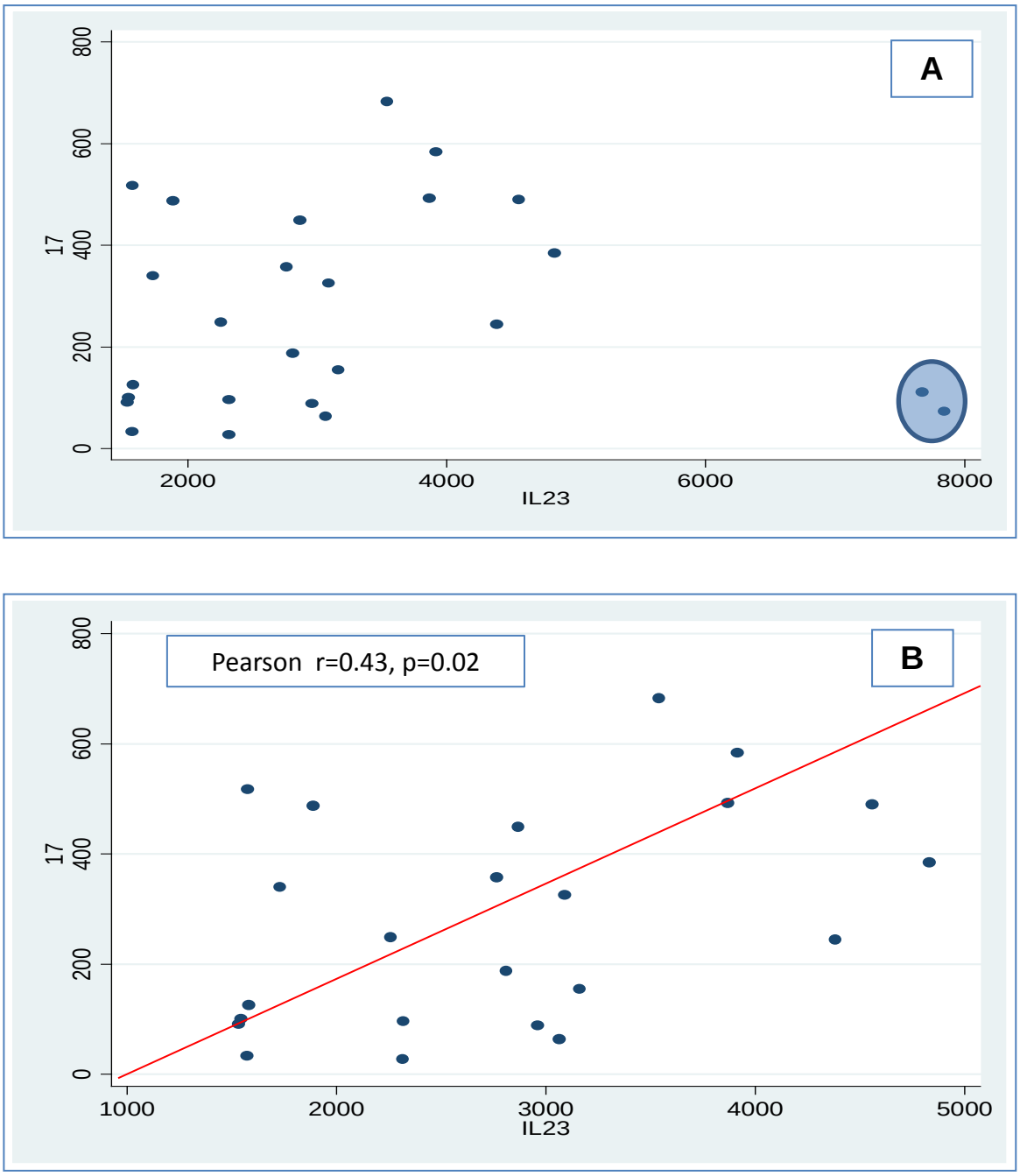


Figure 6.6: Scatter plot (with and without outliers) for correlation betweenIL-17A and IL-23 in GPA. Graph A: In GPA, two outliers (values more than 2 sd) were noted in the data (within blue circle) Graph B: Upon removal of these outliers (i.e. data in blue circle in graph A), there appear to be a possible significant positive correlation (Pearson r = 0.43, p=0.02) between IL-17A and IL-23 in GPA, and linear regression revealed that for every increase of IL-23 by 8 counts there is a 100 count increase of IL-17A (p=0.04)

6.7 Discussion

In this section of the study, we were able to demonstrate the presence of CD68 and IL-23 in orbital GPA, sarcoidosis and IIOD tissues.

GPA, sarcoidosis and IIOD are chronic inflammatory diseases, thus the presence of CD68 in such tissues was expected. However, CD68 was found to be markedly elevated in GPA compared to IIOD. This suggests that, with the presence of more M1 macrophages i.e. active in inflammation, the inflammatory activity in GPA is more intense compared to IIOD. This may explain the more severe clinical presentation and outcome of GPA compared to IIOD. Macrophages also promote other immune cells to the target site during inflammation; hence, this may explain the more cellular presence seen in the histology of GPA compared to OIDs as previously seen in the early part of our study. It was also noted that the CD68 count was comparable between GPA and sarcoidosis. This again was unsurprising as sarcoidosis is also a chronic granulomatous disease like GPA where macrophage activity and presence predominate.

We were unable to get conclusive staining for CD163, CD204 and AIF1. This again could be due to the quality of the sample tissues that we had obtained or the possibility of protein degradation during paraffin tissue preparation. Furthermore, one should not discount that it is possible that these proteins are not present in these tissues.

The significant increase in IL-23 in GPA compared to both IOID and sarcoidosis is particularly interesting. IL-23 is produced by macrophages and dendritic cells and its role

in inflammation is primarily established as a crucial factor in the development of Th17 and IL-17 cytokine production (Boniface et al., 2008). Nevertheless, IL-23 alone has been shown to induce disease pathology. In one animal study, IL-23 was shown to be critical in inducing arthritis and osteoclast formation, independent of IL-17A, resulting in bone destruction (Adamopoulos et al., 2011). This mechanism may offer an explanation to the feature of bony destruction seen in orbital GPA which is not commonly seen in orbital sarcoidosis or IOID. Nonetheless, this would require verification by further studies. IL-23 has also been linked to disease severity. In AAV, including GPA, patients with elevated levels of IL-23 had more active disease compared to those with low IL-23 (Nogueira et al., 2010).

In autoimmune diseases, IL-23 has been shown to drive pathogenic T cells that induce autoimmune inflammation by expanding self-reacting IL-17A, TNFα and IL-6 producing T cells (Langrish et al., 2005). Indeed the IL-23/IL-17 pathway has been shown to be involved in several autoimmune diseases such as Crohn's disease (McGovern et al., 2009) and Vogt Koyanagi Harada disease (Chi et al., 2007). Serum IL-17 and IL-23 levels have been shown to be significantly more compared to healthy individuals in AAV and remained elevated in a proportion of convalescent patients (Nogueira et al., 2010). To the best of our knowledge, this is the first report to show the presence of IL-17A and IL-23 in inflammatory orbital tissues. In this study we not only demonstrated that IL-23 is present in inflammatory orbital tissues, but that IL-23 is also found to be significantly more in orbital GPA. There is also a probable indication of a positive correlation between IL-23 and IL-17A in our GPA group although clarification with further studies will be required.

6.8 Macrophages staining trials

6.8.1 **CD163staining**

6.8.1.1 Trial with variations in length of exposure with Protein K (antigen retrieval)

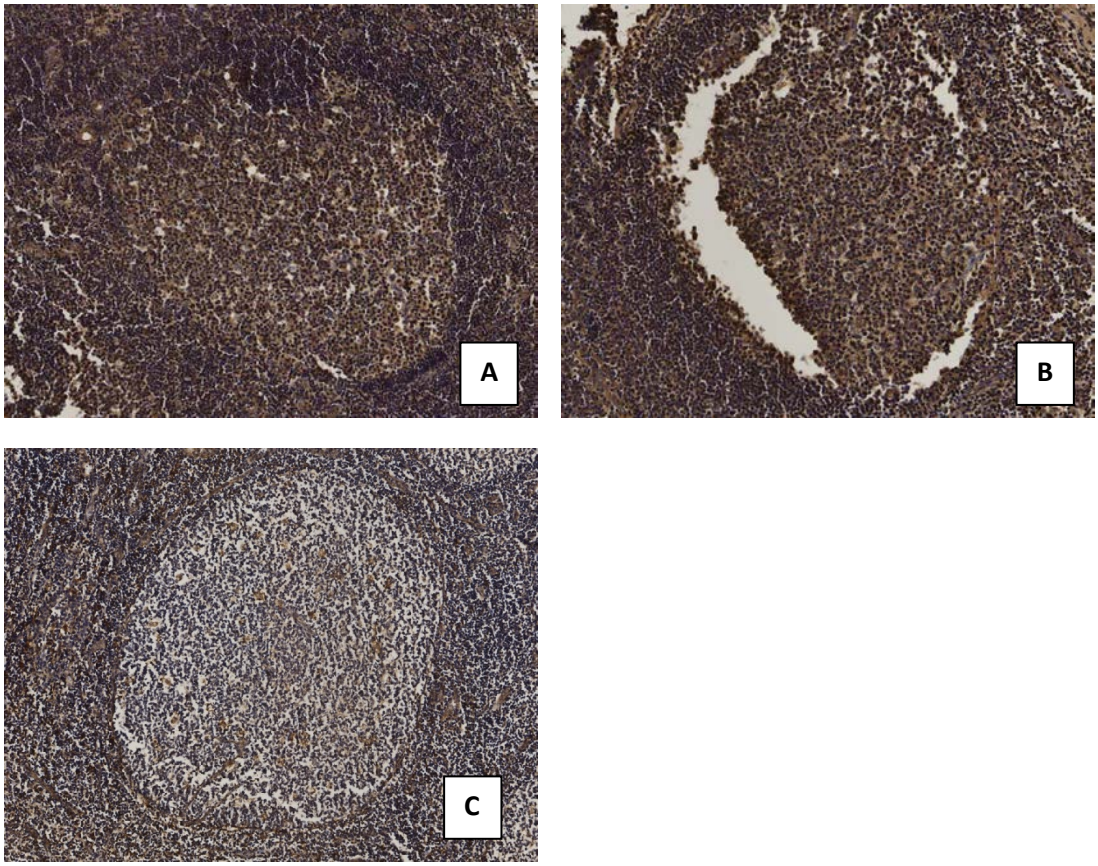


Figure 6.7: Unsuccessful CD163 staining on inflamed tonsil tissues with variations in length of exposure with Protein K as an antigen retrieval. A: Unsuccessful CD163 staining using Protein K for 3 minutes and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40), B: Unsuccessful CD163 staining using Protein K for 5 minutes and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40), c: Unsuccessful CD163 staining using Protein K for 10 minutes and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40)

6.8.1.2 Trial with variations with antibody concentration (using antigen retrieval with protein K for 3 min)

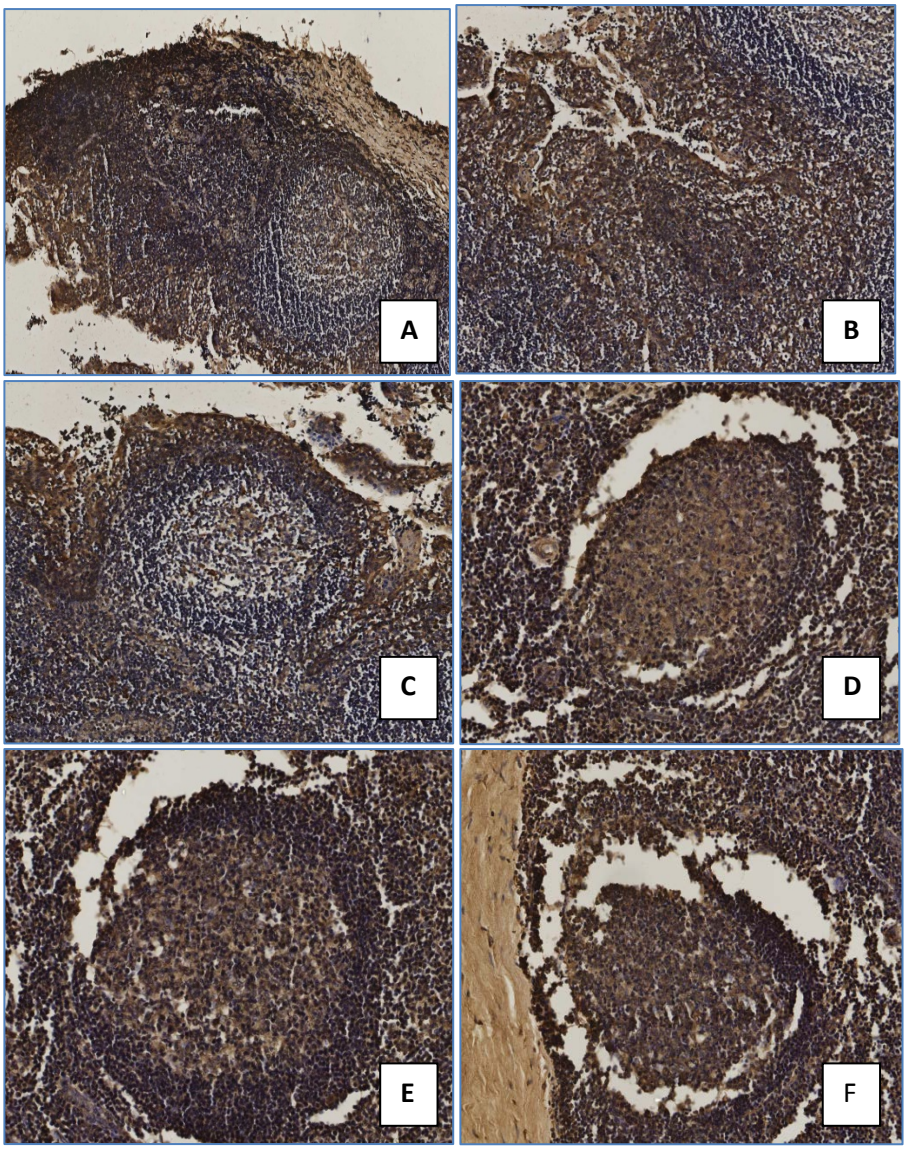


Figure 6.8: Unsuccessful CD163 staining on inflamed tonsil tissues with variations in antibody concentrations A: CD163 staining with antibody concentration of 1:10 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), B: CD163 staining with antibody concentration of 1:20 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), C: CD163 staining with antibody concentration of 1:50 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), C: CD163 staining with antibody concentration of 1:100 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), D: CD163 staining with antibody concentration of 1:200 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), E: CD163 staining with antibody concentration of 1:400 using Protein K for 3 minutes for antigen retrieval. (HRP/DAB+, Rabbit/Mouse.

6.8.2 **CD204**

6.8.2.1 Variation of antibody concentration with sodium citrate pH6 epitome retrieval

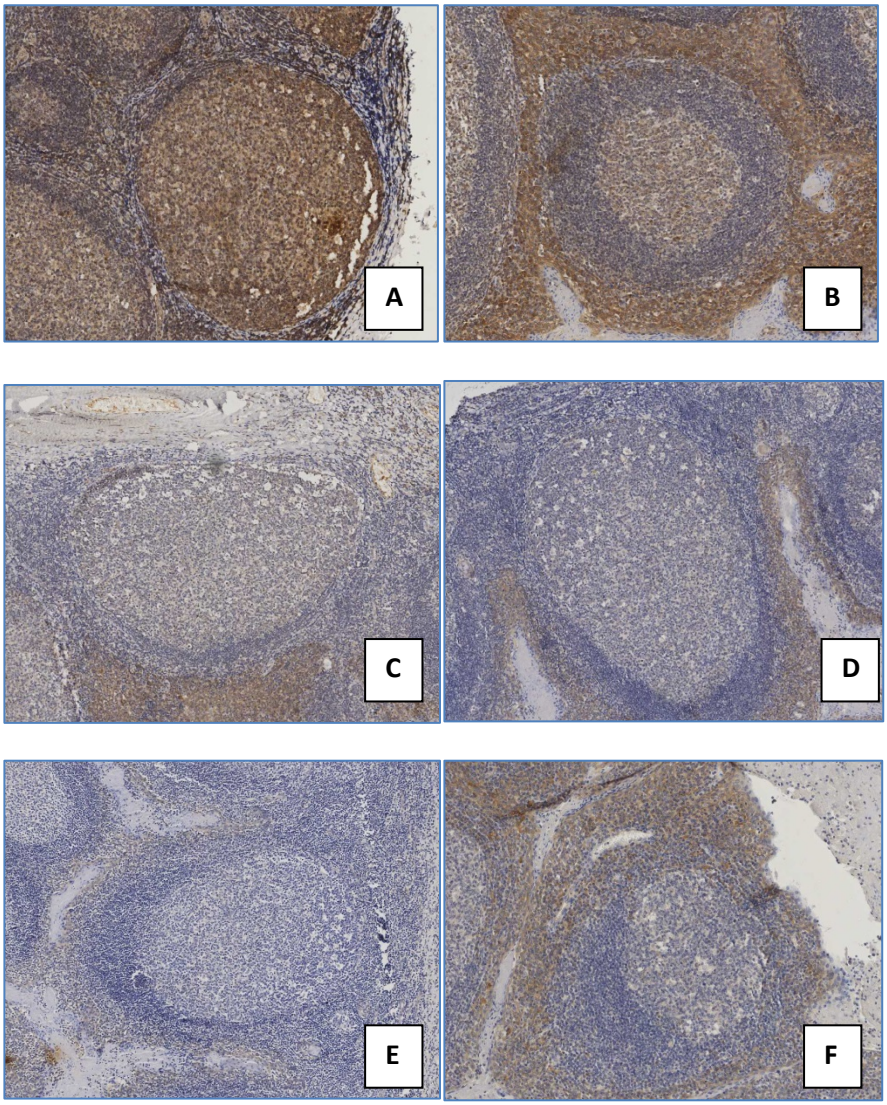


Figure 6.9: Unsuccessful CD204 staining on inflamed tonsil tissues with sodium citrate pH 6.0 and variations in antibody concentrations A: CD204 staining with antibody concentration of 1:10 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), B: CD204 staining with antibody concentration of 1:20 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), C: CD204 staining with antibody concentration of 1:50 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), D: CD204 staining with antibody concentration of 1:100 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), E: CD204 staining with antibody concentration of 1:200 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), F: CD204 staining with antibody concentration of 1:400 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40)

6.8.3 **AIF**

6.8.3.1 Variation with concentration using sodium citrate pH 6.0 for antigen retrieval

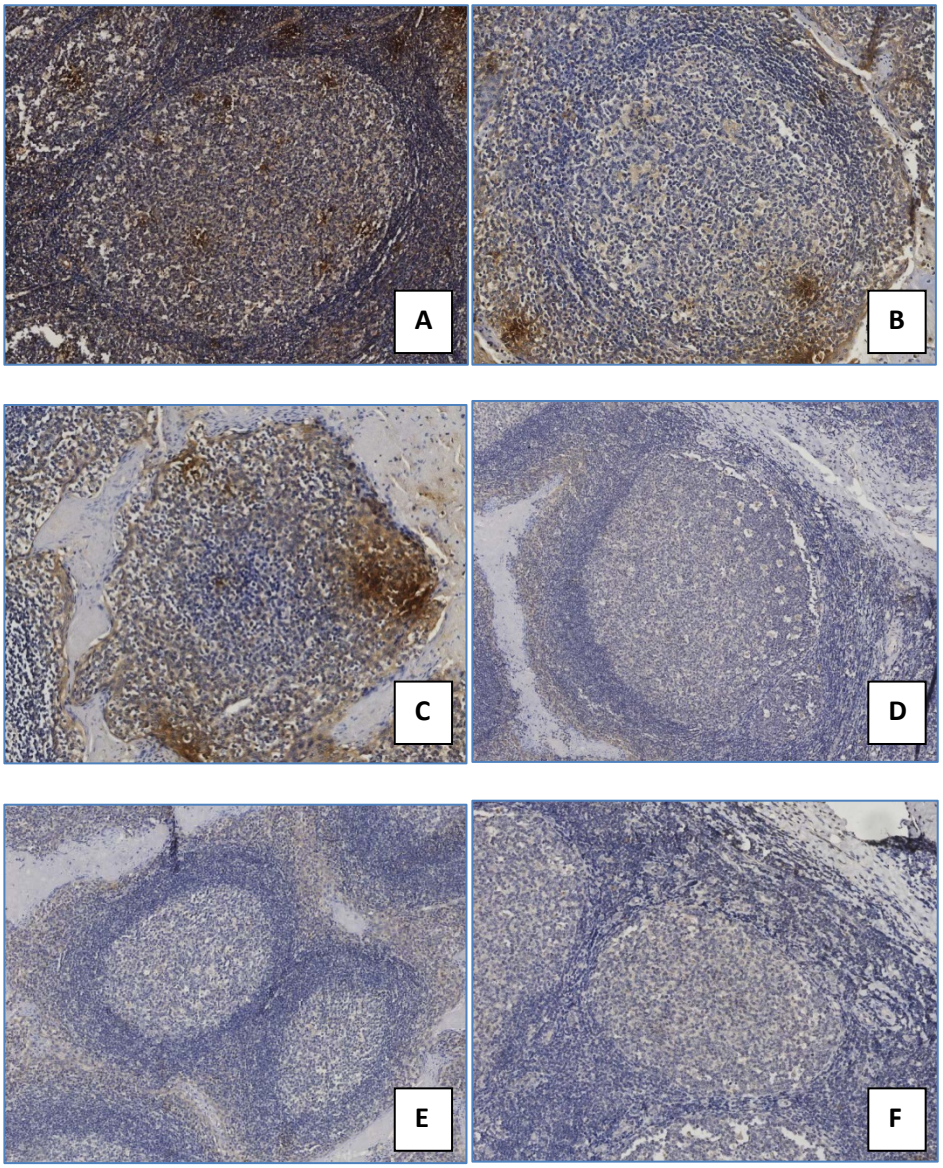


Figure 6.10: Unsuccessful AIF staining on inflamed tonsil tissues with sodium citrate pH 6.0 and variations in antibody concentrations A: AIF-1 staining with antibody concentration of 1:10 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), B: AIF-1 staining with antibody concentration of 1:20 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), C: AIF-1 staining with antibody concentration of 1:50 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), D: AIF-1 staining with antibody concentration of 1:100 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), E: AIF-1 staining with antibody concentration of 1:200 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), F: AIF-1 staining with antibody concentration of 1:400 using sodium citrate pH 6.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40)

6.8.4 *Variation with concentration using Tris/EDTA pH 9.0 for antigen retrieval*

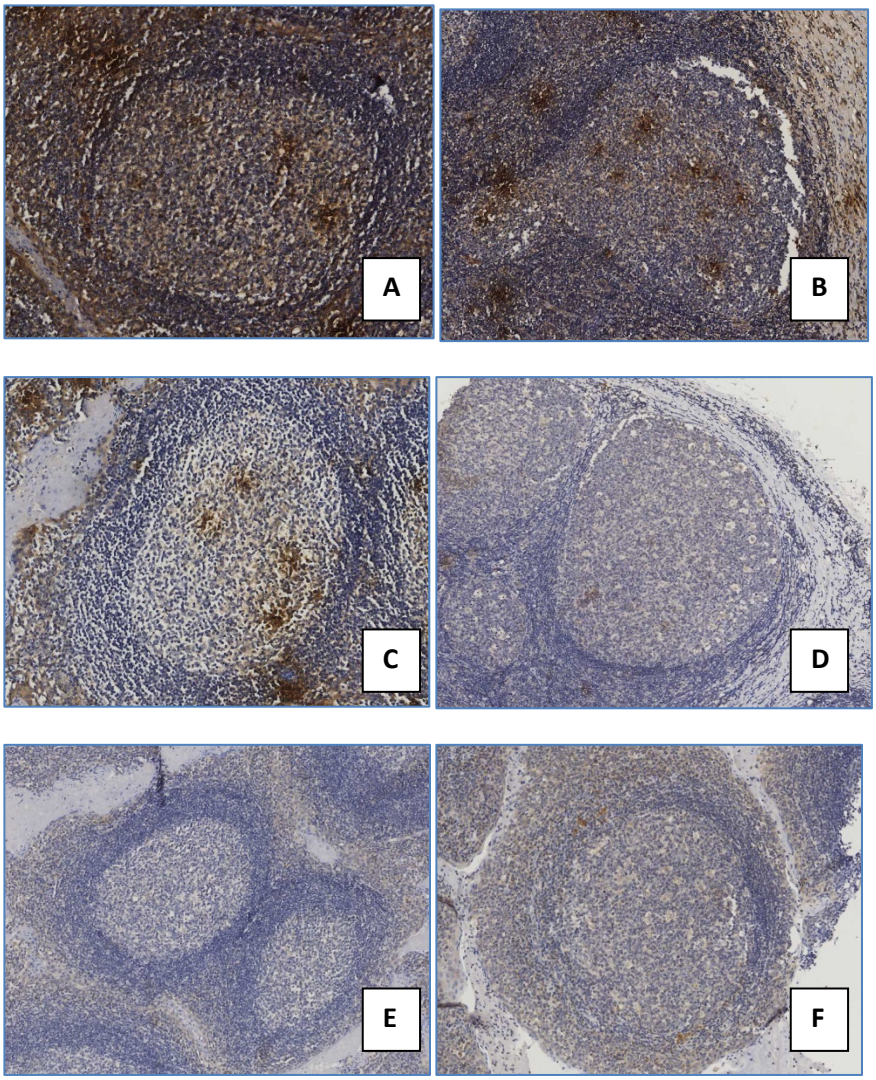


Figure 6.11: Unsuccessful AIF staining on inflamed tonsil tissues with Tris/EDTA pH 9.0 and variations in antibody concentrations. A: AIF-1 staining with antibody concentration of 1:10 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), B: AIF-1 staining with antibody concentration of 1:20 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), C: AIF-1 staining with antibody concentration of 1:50 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), D: AIF-1 staining with antibody concentration of 1:100 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), E: AIF-1 staining with antibody concentration of 1:20 0 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40), F: AIF-1 staining with antibody concentration of 1:400 using Tris/EDTA pH 9.0 for antigen retrieval. (HRP/DAB+, Rabbit/Mouse; x40)

6.8.4.1 Variations in length of exposure with Protein K (in antigen retrieval)

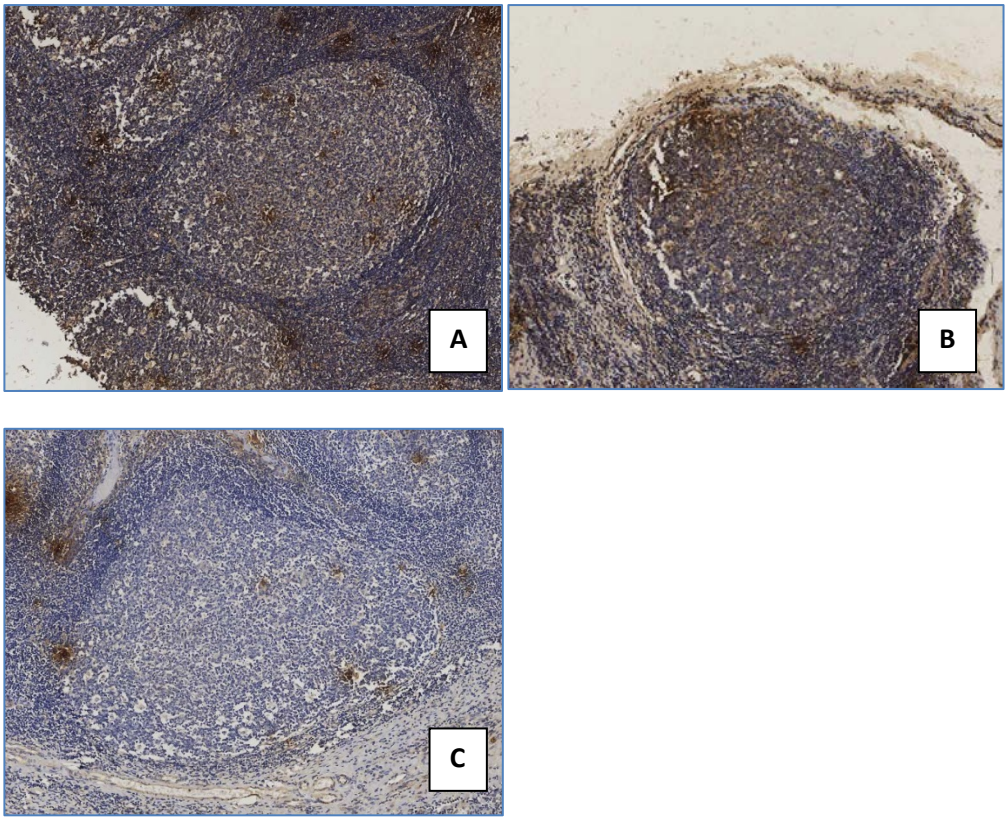


Figure 6.12: Unsuccessful AIF staining on inflamed tonsil tissues with and variations in length of exposure with protein K antibody. A: AIF-1 staining using Protein K for 3 minutes for antigen retrieval and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40), B: AIF-1 staining using Protein K for 5 minutes for antigen retrieval and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40), C: AIF-1 staining using Protein K for 10 minutes for antigen retrieval and antibody concentration of 1:50. (HRP/DAB+, Rabbit/Mouse; x40)

7 Chapter 7: General discussion, Limitation of Study and Future Direction

7.1 General Discussion

7.1.1 Overview

GPA is generally regarded as a rare disease although recent findings have shown an increase in incidence (Watts et al., 2000)(Takala et al., 2008)as well as prevalence(Koldingsnes and Nossent, 2000)(Gibson et al., 2006) of the disease. The disease is also now seen to affect patients at a younger age (Watts et al., 2009). In addition, despite being more common in Caucasians, the disease is now being discovered more and more among other races (Kobayashi et al., 2010)(Kumar et al., 2001)(Chen and Kallenberg, 2010)(Singer et al., 1990)(Wu et al., 2014). GPA can affect any organ system in the body. In ocular GPA, the disease can affect any structure in the eye. The diagnosis of GPA in general, is challenging. This is because the disease manifestation mimics other inflammatory diseases and typically only becomes distinguishable when the disease advances, often in the life threatening stage, or in the case of localised GPA, at the stage when irreversible structural damage or disease progression to the life threatening systemic form, has occurred. Thus, early detection of the disease is crucial. Current advances in the treatments of GPA with immunomodulatory agents and biologics have improved survival rates in GPA dramatically, and structural and organ damage can be limited. This further underscores the need of early diagnosis of the disease.

The diagnosis of GPA relies heavily on a combination of clinical presentations together with investigations such as serological ANCA detection, radiology appearance and histology evaluation. Positive investigation results are often (although not always) more associated with the systemic form of GPA. However, in localised GPA such as orbital GPA, investigations are typically negative. Nevertheless, it is important to note that negative investigations do not preclude the diagnosis of GPA.

When possible, histology plays an important role in the diagnosis of GPA. A combination of granulomatous inflammation associated with geographical necrosis and vasculitis of small to medium size vessels in tissue biopsies, are typically associated with the diagnosis of GPA. Yet, in the absence of these features, particularly in small sample tissues from the nose or orbit, diagnosis becomes inconclusive leading to difficulty in deciding disease management options. Indeed, typical histology appearance are only said to be present in about 30% of cases (Isa et al., 2012). Thus, having a specific biomarker in the histology for GPA to aid early diagnosis would be advantageous. In particular, it would also be of great benefit to find a biomarker in localised GPA that could predict disease outcome (i.e. to progress to remain localised).

Indeed, various cells have been implicated in the pathogenesis of GPA, particularly T and B cells. Nonetheless, many studies are directed towards serum investigations. Very little is done in organ tissue samples and even less in orbital tissues.

7.1.2 **Results in Long-term Outcome of Patients with Orbital GPA**

In the first part of the study, we looked into the long-term outcome (minimum 2 years follow up) of our patients with orbital GPA. The main aim was to identify individuals who progressed from orbital GPA to systemic GPA over time. We discovered that patients with no systemic involvement at the time of presentation, i.e. patients with localised GPA, remained localised and did not progress to the systemic form. The two patients identified as systemic GPA had systemic involvement prior to the orbital presentation. This finding supported the notion that orbital GPA is a separate disease entity and is not necessarily a

part of a widespread systemic spectrum of GPA. In contrast, patients with systemic GPA could progress to cause disease involvement in the orbits as seen in our 2 patients.

We found that patients with orbital symptoms as the initial presentation of GPA (newly diagnosed GPA) had severe clinical symptoms and signs compared to those presented with a recurrence of the disease. Proptosis was the main manifestation of orbital GPA occurring in nearly half the patients (47.2%). This was largely due to the presence of a retrobulbar orbital mass pushing the eye forwards. Lacrimal gland involvement in GPA has not been frequently reported. However, in our study, about 30% of patients had lacrimal gland involvement as the first presentation of the disease.

In general, patients with recurrent orbital GPA came with milder ocular symptoms and signs commonly associated with nasolacrimal duct obstruction (77.7%). Nevertheless, patients with severe visual outcome were among this group of patients as well. Severe visual outcome was associated with patients with severe recurrence of their localised GPA, presenting orbital symptoms such as diplopia and proptosis. Therefore, it appears that, even though orbital symptoms are more severe in cases where the orbit is the initial presentation of GPA, but the long-term visual outcome appears to be good compared to cases of recurrent orbital GPA inflammation. This emphasise the need for early diagnosis and prompt advocation of appropriate treatment in the initial stages of the disease development, to prevent permanent organ damage and functional loss. Interestingly, symptoms of facial pain may be an initial clue for disease recurrence of the orbit in GPA, seen occurring only in the known GPA group in this study.

A small proportion of patients i.e. seven out of 36 of patients (20%), were successfully stabilised with only corticosteroids, however the majority still required second line immunosuppressants along with corticosteroids for disease control. ANCA was only positive in 60% of patients in this study, which is consistent with other reported studies

(Tarabishy et al., 2010). This highlights the poor reliability of this test for the diagnosis of orbital GPA and the need for a more distinctive marker for the disease.

7.1.3 **Histology of Orbital GPA Compared to Other OIDs**

As none of our patients with localised GPA progressed to the systemic form of the disease, we were unable to look for possible prognostic biomarkers in the histology of orbital GPA. However, we proceeded to look for the histological differences between orbital GPA and OIDs.

We found that general cellular activities in orbital GPA were markedly elevated when compared to OIDs. This was seen true when subjective comparison was done with histopathology reports, as well as with objective cellular counts. It is particularly important to note that this comparison was made between the cells in orbital GPA alone with the combined cellular counts from all other OIDs. This highlights the intensity of inflammation that occurs in orbital GPA, reflecting the more severe clinical presentation of the disease compared to OIDs.

Typical histology changes associated with GPA, such as granulomatous inflammation, necrosis and vasculitis, were also features seen in most of our GPA biopsies. However in a number of our GPA tissue samples, some of these characteristic histologic features of GPA were not seen. Despite vasculitis and necrosis being shown to be independently associated with the diagnosis of orbital GPA in this study, necrosis was not observed in the histology of about 31% of our GPA patients (n= 12), and 5 of these patients (13% of total GPA patients) did not have both the features of necrosis and vasculitis in their tissue biopsies. This shows that necrosis and vasculitis are not consistent attributes found in

GPA orbital biopsies thus are unreliable features for diagnosis. In addition, distinct granuloma formation was shown to be inversely associated with the diagnosis of orbital GPA in this study. Therefore, it can be postulated that, orbital biopsies from an orbital inflammatory disease that portrays presence of granuloma in the histology; associated with absent or minimal vasculitic and necrotic features, are indicative of a diagnosis of other OIDs and not orbital GPA.

From this point we wanted to further see if specific immune cell subtypes differ in orbital GPA that could account for the disease development. In particular, we wanted to compare these cell sub-type activities with sarcoidosis and IIOD. These two diseases were chosen on the basis that sarcoidosis share a similar disease process as GPA, which is granulomatous inflammation, and is always the reference point for typical granulomatous diseases. IIOD on the other hand, has similar disease presentations as GPA, both often very difficult to clinically distinguish apart.

7.1.4 **T Cells in GPA**

The role of T cells in the pathogenesis of GPA is well investigated and reported. CD4 has particularly been implicated in the disease development of GPA largely due to the granulomatous nature of the disease. CD8 also has been shown to predominate in kidneys affected by GPA, as well as having a prognostic value. Kidneys with CD8 presence are said to have a worse renal prognosis in GPA compared to kidneys without CD8 presence. However, in our study sample, all main T cell sub-types (Cd4 and CD8) were comparable in all three diseases.

The main element that was found to be significantly elevated in orbital GPA when compared to sarcoidosis and IIOD was cytokine IL-17 ($p<0.001$). In addition, a high IL-17

in tissue biopsies were more likely to be from orbital GPA and unlikely from sarcoidosis and IIOD. IL-17 has indeed recently become the center of attention as the key player for many other auto-immune diseases as well as systemic GPA(Kallenberg, 2011a)(Berden et al., 2009). Our findings were consistent with these results suggesting that IL-17 has a significant role in the pathogenesis of orbital GPA as well. To our knowledge this is the first study to show the presence of IL-17 in orbital inflammatory disease tissues.

We also looked into the presence of CD134, a member of the TNFR superfamily, shown to be present in nasal biopsies affected by GPA. We succeeded in establishing the presence of CD134 in orbital biopsies of GPA, sarcoidosis and IIOD in our study, again, possibly the first study to do so. However, we did not see a significant difference in this cell's presence between the three diseases, indicating that the influence of CD134 in the inflammatory process of these orbital diseases is similar.

Although our study is not focused on the orbital sarcoidosis, we did discover an interesting finding. From the orbital sarcoidosis samples in our study, we did not observe an elevated CD4/CD8 ratio that is usually associated with the diagnosis of the disease using BALF. Furthermore, IL-17 is mainly associated with granuloma formation (Ten Berge et al., 2012), but this was not reflected in our study. IL-17 was markedly more in GPA that is inversely associated with granuloma, and sarcoidosis did not portray an elevated IL-17 despite being associated with granuloma formation. This may indicate that the involvement of T cells in orbital sarcoidosis may somewhat differ, although further investigations would be required to confirm this.

7.1.5 **B Cells in GPA**

B cells were found in all sample tissues from all three diseases. This affirms the effectiveness of Rituximab as a treatment option for these diseases. We were unfortunately unable to properly investigate the presence and extent of BAFF in these orbital diseases. However, the presence of BAFF-R was markedly higher in orbital GPA than sarcoidosis and IIOD. BAFF activity is indeed dependent on the presence of B cells as well as BAFF receptors. BAFF-R is one of the TNF receptors with a high affinity for BAFF. Due to this, it could be concluded that the high BAFF-R level in GPA could indirectly suggest a high BAFF activity as well. The function of BAFF is to prolong the survival of B cells as well as maintain its activity. Increased activities of B cells have been associated with auto-immune diseases presumably from the over-production of auto-immune antibodies, including ANCA. Therefore, although CD20 B cells are similar in all three orbital inflammation, it is possible that in GPA, the more severe presentation of the disease is due to the higher inflammatory activity as a result of longer surviving B cells with a more sustained inflammatory activity, influenced by BAFF.

7.1.6 **Macrophages in GPA**

CD68 and IL-23 was seen present in all three orbital diseases. However, CD68 were only significantly higher in GPA when compared to IIOD. The presence of CD68 was comparable between GPA and sarcoidosis. Indeed, both GPA and sarcoidosis are described as granulomatous diseases and not typically a characteristic of IIOD, although some granulomatous changes can be observed in this disease. Thus, the low CD68 count in orbital IIOD compared to GPA was not unexpected. We were unfortunately unable to

successfully determine the role of M2 macrophages (CD163 and CD204) as well as cytokine AIF1 in GPA, sarcoidosis or IIOD.

The main finding in this section of the study was the presence of IL-23.To the best of our knowledge; this is the first evidence of IL-23 presence in orbital tissues. More importantly, IL-23 was found to be significantly more in orbital GPA compared to IIOD and sarcoidosis. In addition, we calculated that the high IL-23 count in tissue biopsies was 9 times more likely to be from orbital GPA compared to IIOD, and not likely from sarcoidosis. IL-23 indeed has been established in disease development in auto-immune diseases like GPA (Nogueira et al., 2010). IL-23 is one of the key factors in IL-17 production via Th17 (Boniface et al., 2008), thus indirectly influences an inflammatory reaction. It has been shown to be elevated in serum AAVs including GPA. Although there is a suggestion of a possible correlation between IL-17 and IL-23 in our study, we were not able to conclusively prove it.

Apart from its association with IL-17, IL-23 itself has also been independently linked to disease development i.e. inducing arthritis and bone destruction (Adamopoulos et al., 2011). This may explain the bony destruction seen in orbital imaging and cartilage destruction in the nose (saddle nose deformity) seen in GPA but not in IIOD or sarcoidosis. Indeed, in orbital imaging, nasal symptoms, radiological evidence of orbital and paranasal bony destruction is strongly associated with the diagnosis of orbital GPA.

7.1.7 **Overall Conclusion**

The main aim of this study was to attempt to identify a biomarker that could be used to predict disease outcome in orbital GPA by comparing the histology of patients whose

localised disease progressed to the systemic form of GPA; to those whose disease remained localised in the orbit. We found that in our sample population, patients who presented with localised orbital GPA with no systemic disease and did not progress to involve other organ systems. Thus orbital GPA might be regarded as a distinct disease entity associated with the systemic disease. Early recognition of this disease and prompt appropriate treatment is key in the prevention of permanent ocular damage and functional loss. ANCA serology is an unreliable diagnostic tool for the diagnosis of orbital GPA.

Histology examination none the less showed a difference between orbital GPA biopsy and biopsies of OIDs. GPA biopsies portrayed a more increased and intense inflammatory activity compared to OIDs evidenced by higher immune cell counts and tissue changes seen. Necrosis and vasculitis in particular were associated with the diagnosis of orbital GPA in H&E tissue staining but it is important to note that a proportion of the GPA tissue samples did not displayed these changes, making them an unreliable disease indicator. Immunohistochemical staining showed that the main differences found in immune cells activity, between GPA, IIOD and sarcoidosis biopsies, were not among the main immune cell subtypes, such as T and B cell subtypes (e.g. CD3, CD4, CD20 etc.). Instead, significant differences were seen within the cytokine activities. IL-17, BAFF-R (most likely reflecting BAFF activity) and IL-23 cytokines were found significantly more in orbital GPA tissues compared to sarcoidosis and IIOD. The IL-23/IL-17 axis may play a role in the disease development of GPA although a positive correlation between these two cytokines was notable to be established in our sample population. Also, B cells survival and activity appear to be prolonged in GPA, via the influence of BAFF, which may explain the clinical severity of the disease compared to IIOD and sarcoidosis. The significant levels of these cytokines present in GPA compared to IIOD and sarcoidosis suggests that, to some extent, cytokines IL-17, IL-23 and BAFF in orbital biopsies may have a diagnostic value for

the diagnosis of orbital GPA. Therefore, in an orbital biopsy, the presence of these cytokines within the tissues, together with positive clinical correlations, may indicate a higher possibility for the diagnosis of orbital GPA rather than IIOD and sarcoidosis.

7.2 Limitation of Our Study

Our study has some limitations. Firstly, all specimens used were archived tissue biopsies. This may impact the quality of the tissue staining that was done, although we succeeded to produce positive staining and obtain results for most of the target cells and proteins we aimed for. Secondly, our study only demonstrates the presence of these cells and cytokines in these tissues but do not demonstrate the active role or mechanism of action of these entities in the disease development. In addition, performing double staining, fluorescence staining or in situ hybridisation would be helpful to properly identify the cell origin of these proteins tested. Correlation between the cytokine count and clinical outcome would also have been of benefit to provide more information on the influence of these entities in the disease process in orbital GPA.

7.3 Future Directions

In the process of this study we discovered that orbital GPA is a distinct disease entity and may not have systemic involvement, suggesting a favourable survival outcome for patients with orbital GPA. Early diagnosis and prompt treatment can prevent permanent organ damage and visual loss. Histology shows that the immune activities in orbital GPA biopsies are more marked compared to other OIDs, and together with the typical clinical course of the disease, this suggests a distinct mechanism of action in its diseases

development. Therefore continuous studies looking into the pathogenesis of this disease are necessary and important.

We were successful in showing that there is a difference in the activity of immune cells and cytokines between GPA and IIOD as well as with sarcoidosis. We noted that the significant differences were observed not among the main inflammatory cells subtypes; such as CD4, CD8 and CD20, but in other biological factors that influence inflammation such as cytokine IL-17, IL-23 and indirectly BAFF; by the presence of BAFF-R. Thus it is hoped that further studies could follow on from this where, future investigations should be directed towards looking into the function of proteins such as IL-17 and IL-23 in the pathogenesis of GPA. A prospective study of a similar nature i.e. comparing between GPA and OIDs, using fresh tissues biopsies, taken at or nearing the time of disease presentation, while inflammation is still active would be the best way forward. Ideally, biopsy should be done prior to advocating immunosuppressants to display the true picture and activity of the inflammatory cells. In addition, staining for cells with IL-17 receptors and IL-23 receptors instead of the IL-17 and IL-23 cytokines would more likely yield better stainings and results. Following from that, hopefully, better understanding of the disease process in orbital GPA and GPA in general, will be achieved.

This study hopefully, may also assist physicians in the future management decisions for patients with orbital GPA. The significant role of BAFF and B cells in GPA biopsies affirms the effectiveness of medications that target B cells lymphocytes such as Rituximab. The use of this treatment in GPA could be considered earlier in disease development, and not just for refractory cases, particularly in cases where GPA is affecting life threatening organs.

Lastly, this study may hopefully lead to the development of better targeted treatment options for GPA in the future. Drugs targeting the IL-17 pathway or theIL-23/IL-17 axis; that possibly have a significant role in the disease process could be an effective mode of treatment for this disease. This not only will provide another treatment option in the management of GPA, but may also potentially be lifesaving.

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9 Appendix

9.1 Appendix A

9.1.1 Cells and tissue change examples and descriptions on H&E stained tissues

9.1.1.1 Lymphocytes

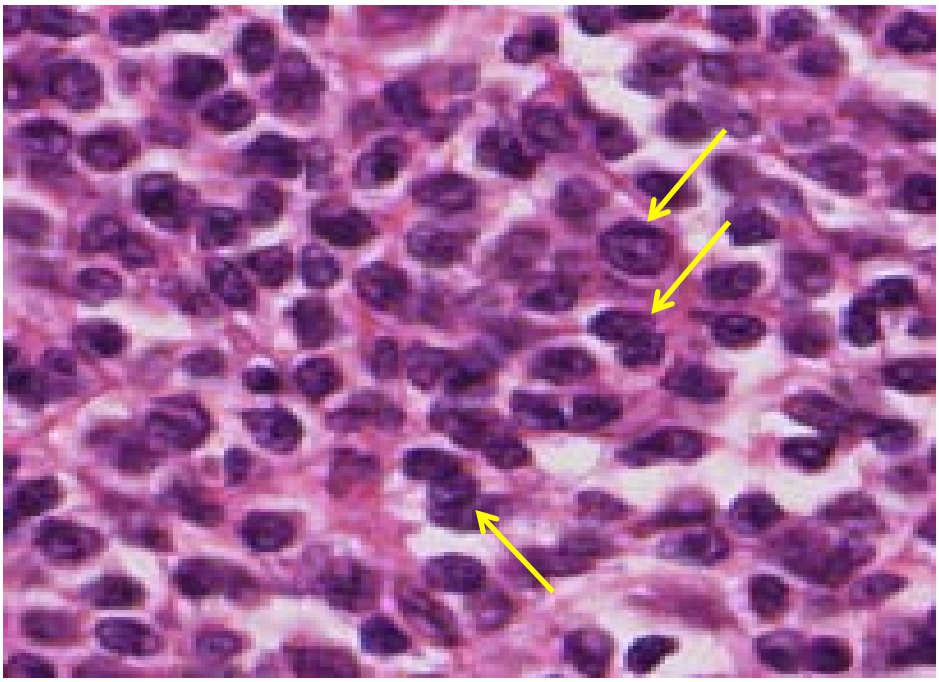

Figure 9.1: Lymphocytes (arrows)

Lymphocytes are white blood cells. They make up about 20-50% of total white blood cells in the human body. They originate from the bone marrow.

Lymphocytes are made up of 3 main cells (agranulocytes) type namely T cells, B cells and natural killer cells. Microscopically and on H&E staining, a normal lymphocyte is distinct from other white blood cells by having a large, dark-staining nucleus with little to no eosinophilic cytoplasm. This coarse, dense lymphocyte nucleus is approximately 7µm in

diameter, about the size of a red blood cell. Occasionally a clear halo-like perinuclear zone around the nucleus is seen in some lymphocytes or could portray a small clear zone to one side of the nucleus. It is not possible to distinguish between T cells and B cells in H&E stain. Lymphocyte phenotypes can be determined via their specific cell surface protein such as cluster of differentiation (CD) markers like CD4 T cells and CD20 B cells, or by the production of particular proteins like cytokines.

Lymphocytes play an integral part of the immune system where their main function is to defend the body against pathogens such as bacteria, parasites and viruses; cancer cells as well as foreign materials. Apart from circulating in the blood and lymph fluid, lymphocytes can also be found in organs such as the spleen, thymus, tonsils, liver, bone marrow and lymph nodes. The immunity against antigens by lymphocytes is achieved by 2 main immune responses which are the cell mediated immunity; mainly by T cells function, and humoral (antibody-driven) adaptive immunity, mainly by B cells. In general cells, mediated immunity focuses on the active destruction of infected or cancerous cells, while the humoral immunity focuses on identifying antigens prior to cell infection and antibody production.

9.1.1.2 Plasma cells

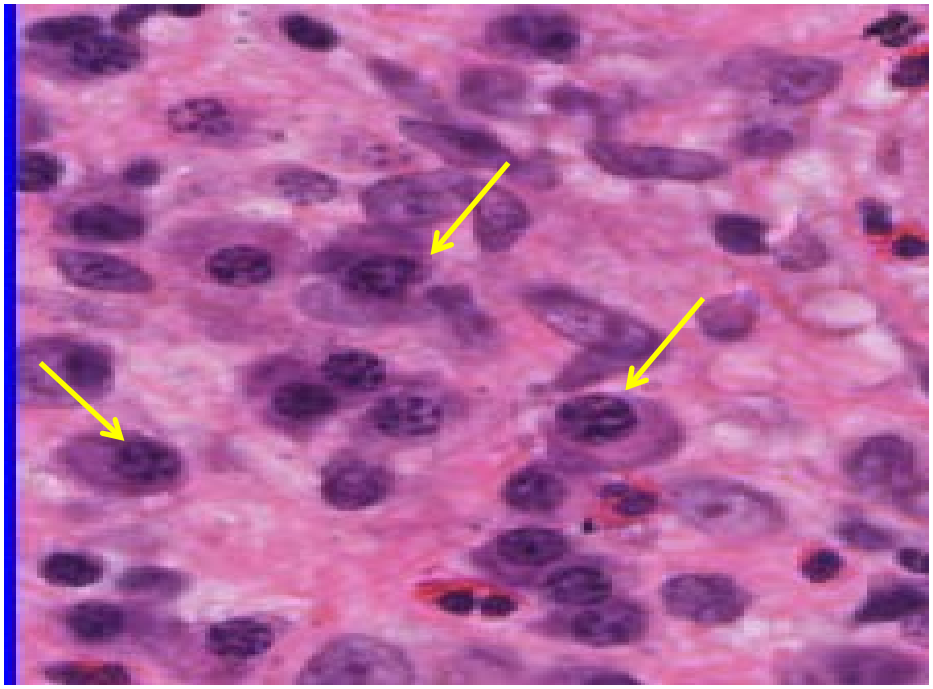

Figure 9.2: Plasma cells (arrows)

Plasma cells are also known as plasma B cells, plasmocytes, and effector B cells. They are differentiated mature B cells that are specialised for the production of large volumes of antibody (immunoglobulins). Plasma cells originate from the bone marrow and move freely in connective tissues. In the peripheral blood plasma cells constitute about 0.1-3% leucocyte count in bone marrow. Plasma cells have distinctive microscopic features which are easily identified on H&E tissue staining. Morphologically, they are typically large lymphocytes which are oval in shape measuring 10-20µm. They have a considerable nucleus-to-cytoplasm ratio, where the cytoplasm is deeply basophilic, reflecting the ribosomal content of the cell, and an eccentric nucleus. The nucleus contains within it heterochromatin that alternates with light areas giving rise to a cartwheel or clock face feature which is characteristic of the cell. Plasma cells produce antibodies that are

released locally in tissues or in the blood circulation and are essential for defence against infections.

9.1.1.3 Eosinophils

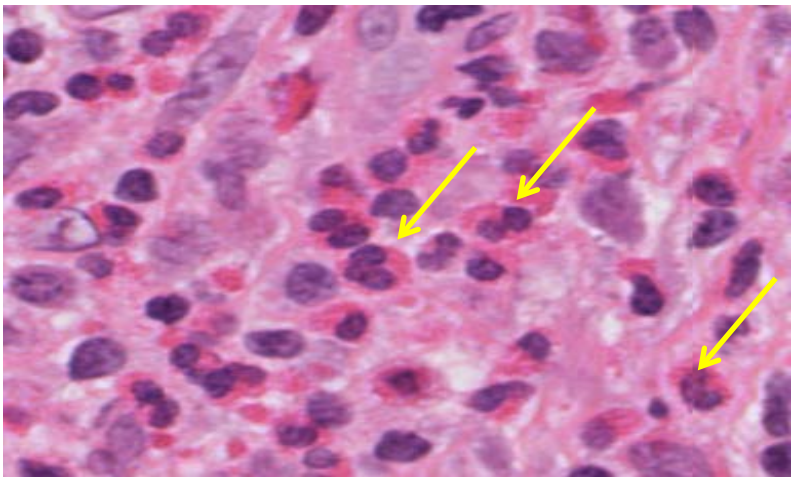

Figure 9.3: EosInophils (arrows)

Eosinophils are granulocytic white blood cells. Originating from the bone marrow, they form about 2-6% of the human white blood cells. They are about 12-17 µm in size based on microscopic examination. They are acid loving, hence the term “eosinophilic”, a property that is responsible for the brick red appearance of the cell on staining with eosin. Eosin staining on these cells is also mainly concentrated in the small granules within the eosinophil cytoplasm; thus making the cell appear granulated microscopically. These granules contain histamines and cytotoxic proteins and play a role in pathogen eradication and inflammation. Apart from these features, eosinophils are also characterised as having lobed nucleus, either two or four lobes. In the circulation, they persist for about 8-12 hours but in tissues they can survive for up to12 days even without active stimulation. Eosinophils can be found in the thymus, lower gastrointestinal tract, uterus, ovary, spleen and lymph node. Their presence in other organs is usually associated with disease.

Development and maturation of eosinophils is in response to cytokines IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF). One of the main functions of eosinophils is during parasitic infection. During invasion, these cells can degranulate releasing the cytotoxic proteins and together with IgE antibody, eradicate the pathogen. Other functions include destruction of tumour cells, regulating other immune cells functions such T and B lymphocytes via becoming an antigen presenting cell, and also promote damage tissue repair. Together with mast cells, they are also play a part in allergic reactions.

9.1.1.4 Neutrophils and nuclear debris

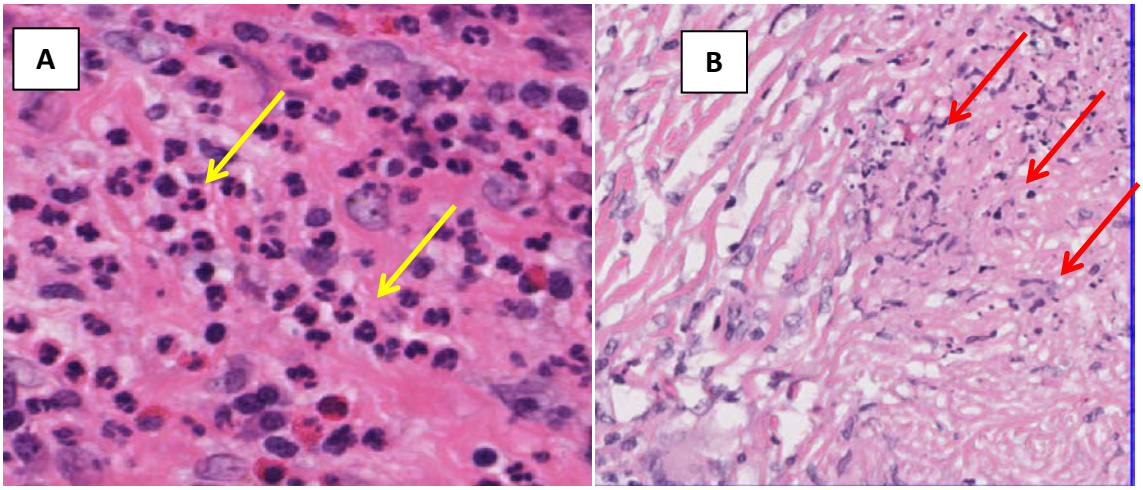

Figure 9.4: A: Neutrophils (yellow arrows) and B:Nuclear debris (red arrows)

Neutrophil granulocytes; also known as neutrophils are formed from stem cells in the bone marrow. They make up the majority of the phagocytes in humans, constituting 40% to 75% of total white blood cells. Together with eosinophils and basophils, they form the polymorphonuclear cells characterised by their multilobulated nucleuses. Generally, neutrophils have an average diameter of 8-15 µm and contain a nucleus divided into 2–5 lobes. The separated lobes are connected to one another by chromatin. The name

neutrophil derives from staining characteristics on haematoxylin and eosin (H&E) histological or cytological preparations. In contrast to eosinophils which stain bright red and basophils that stains dark blue, neutrophils stain with a neutral pink hue. Neutrophils are short-lived and highly motile. In the initial phase of inflammation i.e. acute inflammation, either due to infection, trauma or cancer; neutrophils are seen as the first and main responders migrating from blood vessels to the site of attack or injury. The migration of neutrophils through interstitial tissues is influenced by chemical signals such as IL8, C5a and leukotriene B4, where this process is termed as chemotaxis. Neutrophils recruitment to the site of injury occurs within minutes, and these cells are the hallmark of acute inflammation. Nuclear debris is believed to be fragments of neutrophils left after degradation during an inflammatory process. This however this remains uncertain. In pus, neutrophils are the predominant cells seen, accounting for its whitish/yellowish appearance.

9.1.1.5 Mast cells

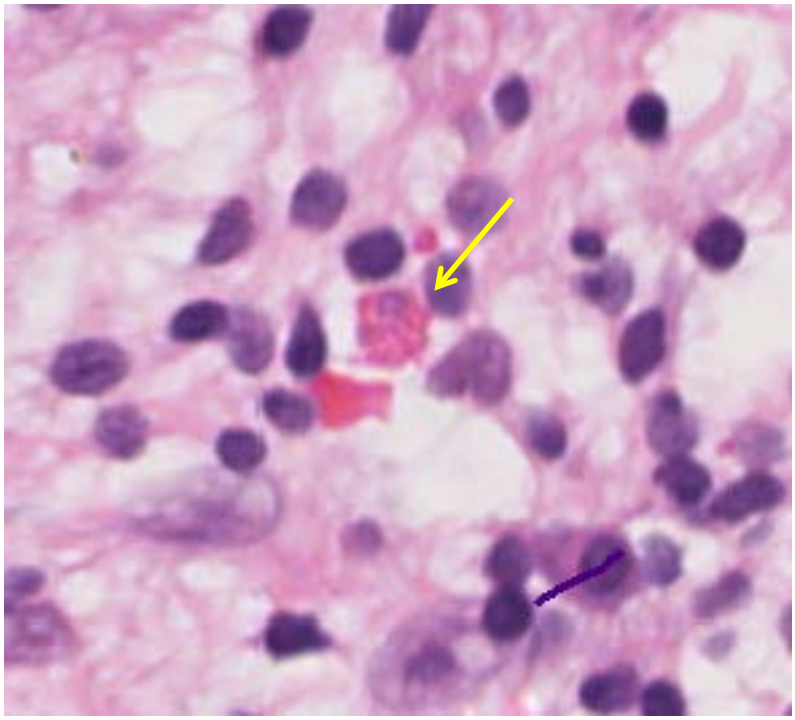

Figure 9.5: Mast cells (arrow)

Mast cell is a granulocytic cell found in mast tissues and mainly found surrounding blood vessels and nerves. They appear mostly in tissue layers with close contact to the external environment such as the skin, intestinal lining, lung mucosa, mouth, nose and also conjunctiva. Mast cells have similar properties to basophils from the white blood cell family in that both contain granulated cells within them containing histamine and heparin. Mast cells are generally known for their role in allergies and anaphylaxis reaction via interaction with immunoglobulin E (IgE) resulting in histamine release. Apart from this, they also have a protective role particularly against intestinal worn infestations.

9.1.1.6 Macrophages and giant cells

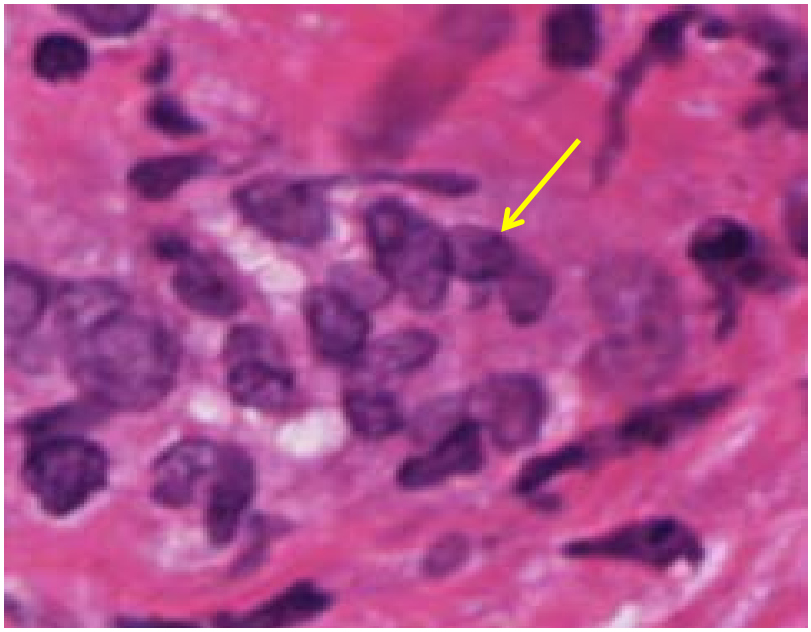

Figure 9.6: Macrophages in the form of epithelioid cells forming a multinucleated giant cell

Macrophages are large specialised cells of the immune system. It has the ability to recognise, engulf and ingest pathogens and target cells. Macrophages are differentiated circulating monocytes that change into macrophages upon migration and entry into affected tissues or organs. Thus macrophages provide the first line of defence from infection in tissues. Macrophages are about 21µm in size and can survive for several months. Activated macrophages can transform in shape resembling epithelial cells known as epithelioid cells. These cells appear elongated with pale granular eosinophilic cytoplasm with an ovoid nucleus. The shape and contour of epithelioid cells are non-distinct but these cells often aggregate into one another forming giant cells. Further organisation of these cells is the basis of granuloma formation.

9.1.1.7 Granuloma

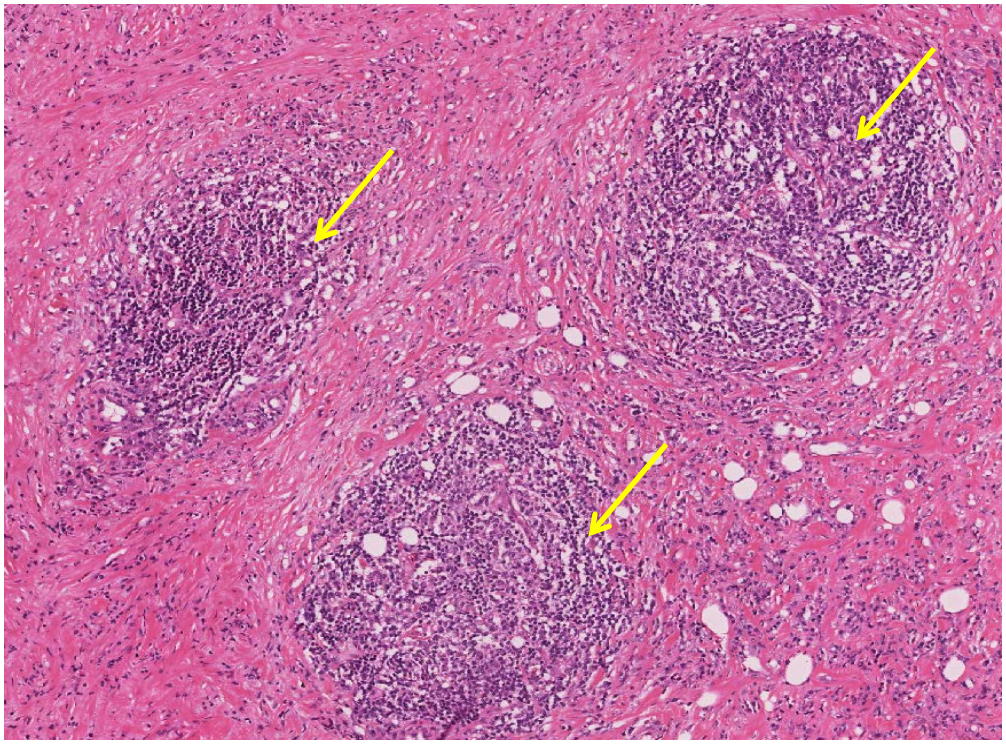

Figure 9.7: Granuloma (arrows)

Granulomas are an organised collection of immune cells, predominantly constituting macrophages. It is an immune response to attempt to wall off foreign materials in the body which were not or cannot be eliminated. These foreign materials include bacteria, fungi or foreign materials such as suture fragments. All granulomas, regardless of cause, could contain a number of other immune cells and matrix. These other immune cells include neutrophils, lymphocytes, eosinophils, giant cells, fibroblasts and may also have fibrosis formation within it. The additional cells may sometime indicate the cause of the granuloma formation e.g. neutrophil presence in aspiration pneumonia, eosinophils in fungal infections, etc. Granulomas can be present in a wide variety of disease, in either infectious or non-infectious pathology. In infections, granuloma formations can be seen in tuberculosis. Leprosy, histoplasmosis and catch scratch diseases. Sarcoidosis,

rheumatoid arthritis, GPA and eosinophilic GPA (Churgg-Strauss syndrome) are some non-infectious conditions that could portray granuloma formations in its histology. Granuloma can also show different characteristics morphologically. A necrotic granuloma for example is seen as a granuloma containing dead cells, appearing as a mass of formless debris with no nuclei present. Caseous granuloma is termed for granuloma with a central “cheese like“(caseation) appearance, usually (although not always) seen in tuberculosis infection.

9.1.1.8 Vasculitis

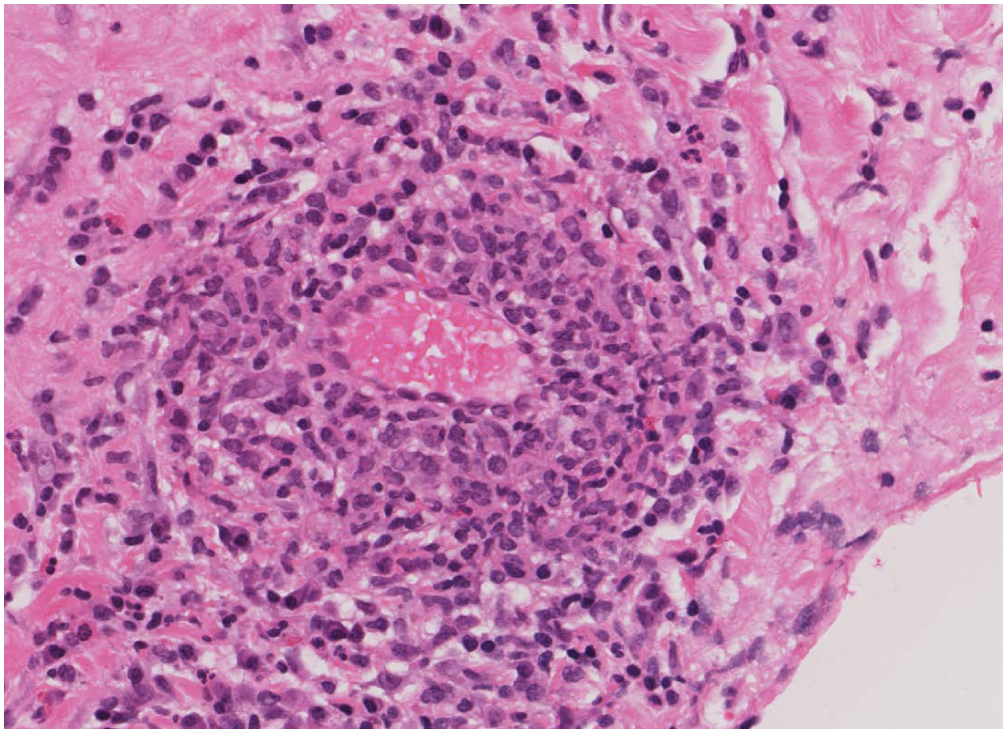

Figure 9.8: Vasculitis

Vasculitis infers inflammation and damage to blood vessel walls. The blood vessel involved can either be capillaries, venules, arterioles, arteries and even large veins. Pathologically, vasculitis is divided according to the size of the vessels involved namely,

small vessel vasculitis, medium vessel vasculitis and large vessel vasculitis. In small vessel vasculitis, the pathology can further be associated with either a positive ANCA association such as in GPA and MAP or a negative ANCA association such as Henoch-Sconlein purpura, infective induced or drug related hypersensitivity vasculitis. Diseases associated with medium vessel vasculitis include PAN and Kawasaki disease although in GPA, vasculitis if medium size vessels have been noted. Large vessel vasculitis is seen in Takayasu disease and giant cell arteritis.

There are different types of inflammation that can occur in the walls of the affected vessel. Neutrophilic vasculitis results in necrosis of the blood vessel wall (termed necrotising vasculitis) where fibrin deposition (fibrinoid necrosis) and neutrophil infiltrations are seen. In giant cell vasculitis affecting large vessels, histiocytic (macrophage) infiltrations are seen in the vessel wall with formation of multinucleated giant cells observed. Granulomatous vasculitis is associated with histiocytic granuloma formation adjacent to the damaged vessels and lymphocytic vasculitis demonstrates vessel wall infiltration with lymphoid cells.

9.1.1.9 Fibrosis

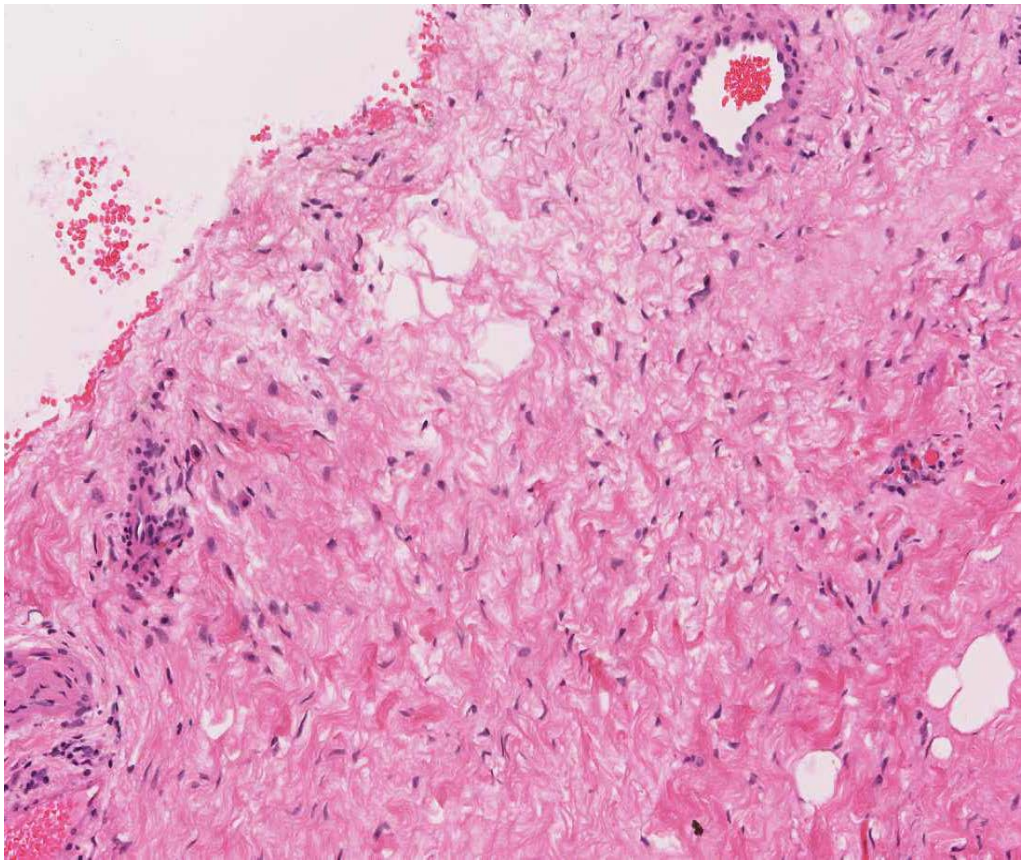

Figure 9.9: Fibrosis

Fibrosis is the generally a formation of excess fibrous connective tissue in an organ or tissue. It is usually a reparative sequelae of tissue damage; either from trauma, inflammation or infection. The process is similar to scarring where it involves laying down collagen, glycosaminoglycan as well as other connective tissues. TNF Beta is mainly the stimulating protein for this process where it stimulates the activation and proliferation of fibroblast. Activated fibroblasts will in turn deposit connective tissues. Fibrosis is usually benign however it can cause structural damage and organ dysfunction.

9.1.1.10 Necrosis

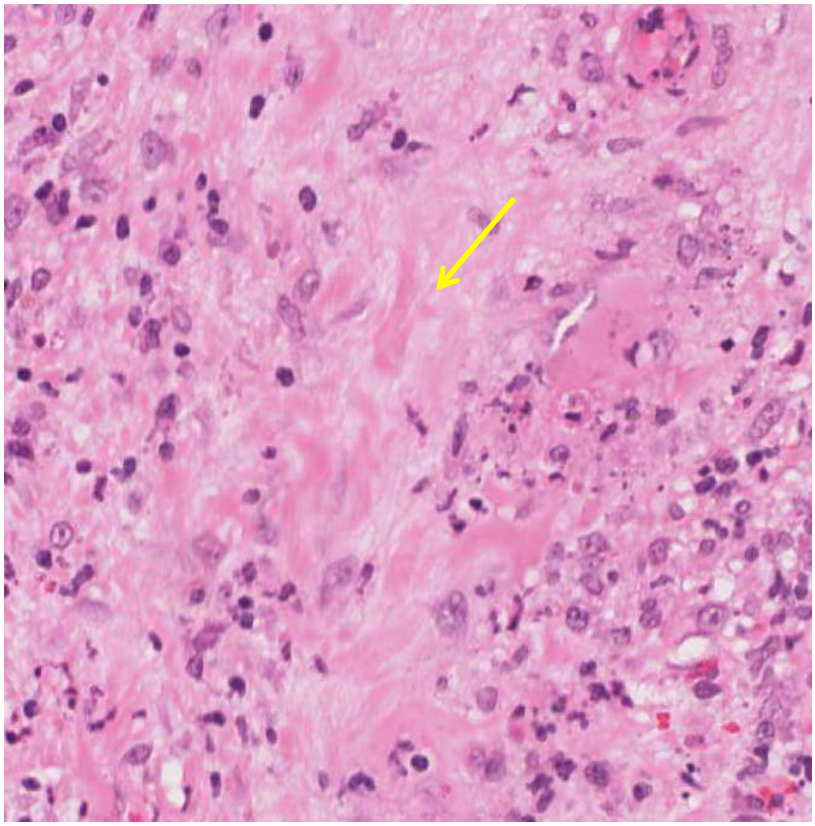

Figure 9.10: Necrosis (arrow)

Necrosis is an irreversible cell injury resulting in cell and tissue death. Factors that could cause tissue necrosis include ischaemia, physical agents like trauma, chemical agents like toxins and immunological injury during infections and inflammation. Histologically necrosis is the combination of morphologic changes following cell death in living tissues or organ. The course of necrosis involves denaturation of intracellular proteins as well as digestion of cells by enzymes. Changes that are seen are usually observed in both cytoplasm and nucleus. The cytoplasm generally becomes homogenously glassy in appearance with vacuolation. There is also calcification of dead cells and increased eosinophils in the cytoplasm as well as appearance of myelin figures and generation of calcium soaps. Nuclear changes undergo three main changes which are karyolysis, where there is fading

of basophilia of chromatin due to DNA activity; pyknosis, which is nuclear shrinkage and increased basophilia; and karyorrhexis, a process of fragmentation of pynknotic nucleus.

There are several types of necrosis namely; coagulative necrosis, liquefactive necrosis, caseous necrosis, fat necrosis, gangrenous necrosis and fibrinoid necrosis. Coagulative necrosis can occur in any tissue except the brain and is generally due to blood loss. Tissue becomes firm and microscopically cells outlines are preserved but appear ghostly and tissues look generally red. Liquefactive necrosis is commonly associated with infection, and also in brain infarcts. Grossly tissues become liquefied and creamy yellow (pus). Microscopically neutrophils are seen to predominate among dead cellular debris. Caseous necrosis is usually associated with tuberculosis. Tissues turn cheese like in appearance and histologically granuloma formations are seen. Fat necrosis occurs in acute pancreatitis. The enzyme lipase contained in pancreatic cells is released during necrosis and splits triglycerides within fat cells. Grossly, chalky white areas resulting from fatty acids and calcium (saponification) combination is seen. On microscope examination, shadow outlines of dead fat calls, which have basophilic features can be observed. Fibrinoid necrosis has been associated with GPA and affects vessels. It consists of a complex of antigens, antibody and fibrins. Microscopically vessel walls appear thickened and pinkish red. Gangrenous necrosis occurs when an entire limb losses blood supply and dies. Skin looks discoloured and dead, and underlying tissues become decomposed. Histologically, coagulative necrosis occurs and in an event where infection sets in, liquefactive necrosis results.

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Ocular Manifestations of Wegener's Granulomatosis

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Expert Rev Ophthalmol. 2011;6(5):541-555.

Abstract

Wegener's granulomatosis (newly renamed granulomatosis with polyangiitis [WG/GPA]) is a granulomatous autoimmune inflammatory disorder of unknown etiology that is associated with anti-neutrophil cytoplasm antibodies. It can involve any organ system, but most commonly affects the respiratory and renal systems and the head and neck, including the eye. The ophthalmic manifestations of WG/GPA are diverse and can affect any of the ocular structures. Despite continuing research, the exact pathogenesis of WG/GPA remains elusive, but there is increasing awareness of the disease, especially as its prevalence and incidence are both on the rise. Unhelpfully, the diagnosis and management of WG/GPA remains a challenge, especially in its limited form, as blood tests, imaging and tissue biopsies are often negative or nonspecific. Nevertheless, the emergence of new treatment regimens for WG/GPA, particularly target-specific drugs such as rituximab, the anti-CD20 antibody, is bringing promise in providing patients with better and potentially safer treatment for the disease.

Introduction

Granulomatosis with polyangiitis (previously known as Wegener's granulomatosis, Wegener's granulomatosis/granulomatosis with polyangiitis [WG/GPA]) is a multisystem granulomatous inflammatory disorder with vasculitic and necrotic manifestations, and is presumed to be of autoimmune origin.[1] It was initially described by Heintz Klinger, a medical student, in 1931, but the disease was later named after a German pathologist, Frederick Wegener, who defined the disorder and clearly distinguished it from other inflammatory diseases such as polyarteritis nodosa.[2,3]

The pathology of WG/GPA is characteristically described as a granulomatous necrotizing inflammation and pauci-immune vasculitis of the small- to medium-sized blood vessels (Figure 1). WG/GPA can affect any organ system but has a tendency to affect the upper and lower respiratory tract system and the kidneys. Together with Churg–Strauss syndrome, microscopic polyarteritis (MPA) and renal limited vasculitis, WG/GPA has been demonstrated to have a strong association with antineutrophil cytoplasm antibody (ANCA), although not all cases may be accompanied with a positive ANCA serology. This group of disorders is known collectively as the ANCA-associated vasculitides (AAV). In the eye, the manifestations of WG/GPA are variable, but can involve any ocular structure. Indeed, ocular involvement occurs in up to 60% of WG/GPA patients and not infrequently forms the initial presentation of the disease.[4]

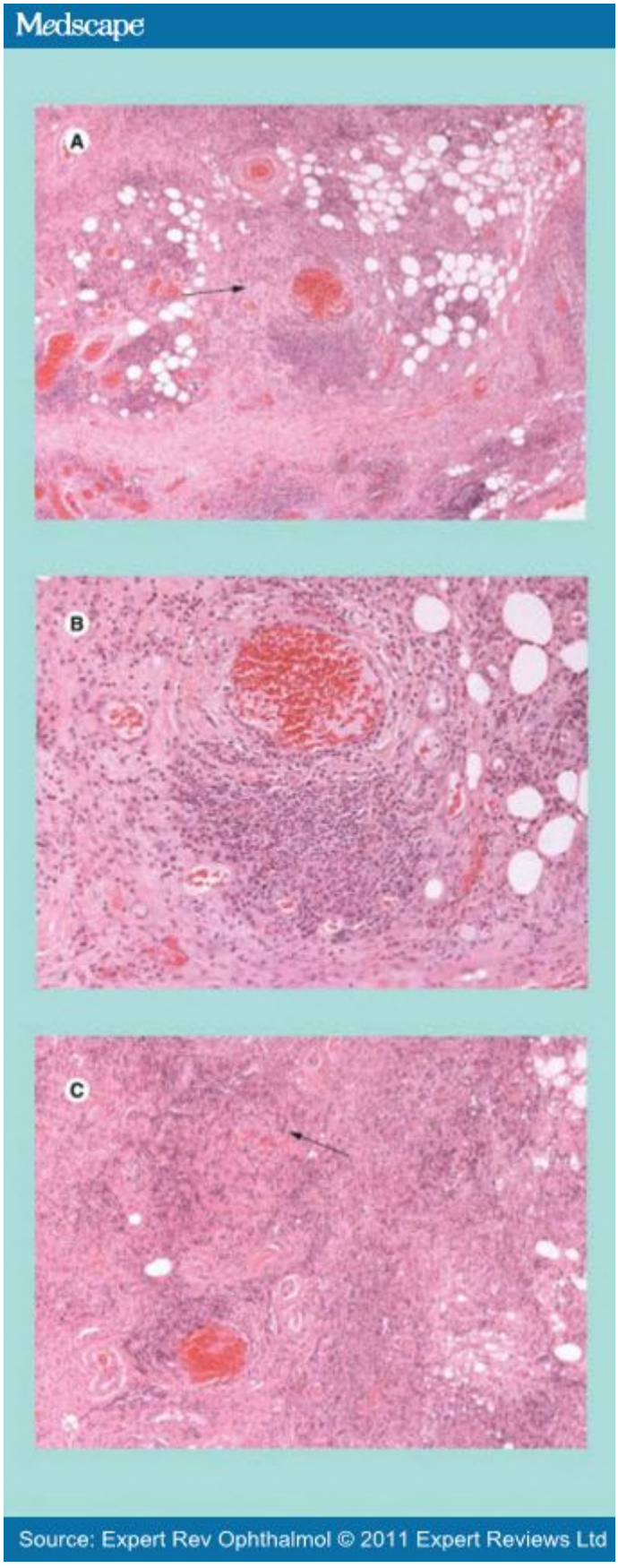
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Figure 1.

Wegener's granulomatosis/granulomatosis with polyangiitis. **(A)** Vasculitis (arrowed) and granulomatous infiltrates are apparent. **(B)** High-power photograph of a vasculitic blood vessel demonstrates neutrophil infiltration of the vascular wall. Eosinophils and lymphocytes are also present. **(C)** Typical granulomatous infiltration is apparent.

Epidemiology

The overall annual incidence of WG/GPA is estimated to be approximately 4–8.8 cases per million, but this varies depending on location; in the UK, the annual incidence of WG/GPA is reported as being at the higher end of the scale at 8.4 cases per million.[5] WG/GPA has been reported throughout the world,[6–9] but is more common among Caucasians,[10] and epidemiological studies have demonstrated an apparent latitude-dependent predisposition, with a decreasing north–south gradient in the northern hemisphere and a reciprocally increasing south–north gradient in the southern hemisphere, although the reasons for this remain unclear.[11–14]

The peak incidence of WG/GPA is seen in the sixth decade of life and there appears to be no gender predilection. Although WG/GPA is considered a relatively rare disease, recent studies have shown that its incidence [11,15] and prevalence [5,11,15] lie on an increasing trend, and it is now affecting patients of a younger age.[5,11,14,16]

General (Systemic) Versus Limited WG/GPA

Wegener's granulomatosis/granulomatosis with polyangiitis has a broad range of clinical presentations and can affect any organ with variable disease severity. Generally, WG/GPA can present in a limited or a general (systemic) form. However, these terms are mainly widely used in clinical trials to classify patients for the purpose of treatment options and in medical literature, but are not commonly used during clinical practice. To date, there is no current consensus on the characteristics that clearly define these two forms.

Limited WG/GPA was first described in 1966 by Carrington and Liebow as the identical clinical onset and pathologic manifestations to the classic form of the disease, except in the absence of renal involvement.[17] The Wegener's Granulomatosis Etanercept Trial (WGET) research group used the term 'limited WG' in their clinical trial for patients fulfilling the American College of Rheumatology (ACR) criteria for WG/GPA, but lacking features that pose immediate threat to either a critical individual organ or to the patient's life.[18] The term 'localized WG/GPA' has also been used in medical trails and literature, although it has not been generally accepted worldwide. In their study, the European League Against Rheumatism (EULAR) group defined localized WG/GPA as individuals with vasculitic features confined only to the upper and/or lower respiratory systems, including the orbit.[19,20] In general, patients with limited WG/GPA are often considered to run a milder course of the disease and usually respond well to less aggressive treatment regimens.[21,22]

General (systemic) WG/GPA, by contrast, reflects a more severe form of the disease with a more widespread organ system involvement. Severe WG in the WGET encompassed patients with significant involvement of vital organs that is potentially life threatening.[18,23]EULAR divided patients with general WG into early systemic, generalized, severe and refractory.[19,20]

Ocular and orbital structure involvement is common in patients with both limited and general forms of WG/GPA, and may be the presenting feature in both. A very limited form of WG/GPA, with localized ophthalmic features only, has also been described.[24] There appears to be no difference between the spectrum of ocular manifestations and incidence of potentially sight-threatening complications seen in either the general and limited forms.[25]

Pathogenesis

The exact pathogenesis of WG/GPA is still unknown, but an interplay of both cellular and humoral immunity are believed to be involved in the disease process. Pathologically, WG/GPA is described as a triad of granulomatous inflammation, vasculitis of small- to medium-sized vessels and necrosis. ANCAs have been demonstrated to have strong associations with WG/GPA as well as with the other AAV. These are a group of IgG antibodies directed against the cytoplasm of neutrophil granulocytes and monocytes and can be detected in the serum by indirect immunofluoresence.

Based on the pattern of staining on ethanol-fixed neutrophils, ANCAs can be divided into two main patterns: cytoplasmic-ANCA (c-ANCA; which show a diffusely granular, cytoplasmic staining pattern and are strongly associated with WG/GPA) and perinuclear-ANCA (p-ANCA), which show a perinuclear staining pattern, mainly associated with MPA. In AAV, ANCA are shown to specifically target the antigens proteinase 3 (PR3), the predominant antigen in WG/GPA, or myeloperoxidase, the predominant antigen in MPA, and these can be detected by ELISA. The exact mechanism by which ANCA are generated is as yet undetermined, but molecular mimicry by viruses or bacteria, drugs, and genetic predisposition, such as the defective allele *PI*Z* on chromosome 14q32.1 [26] and *HLA-DPB1*,[27] have all been associated with WG/GPA and may potentially be possible causes in the development of ANCA in AAV.[28,29]

The etiology and exact role of ANCA in the pathogenesis of WG/GPA is still uncertain, and studies in this area are ongoing. *In vitro* studies have shown that ANCA activate primed neutrophils, promoting their adherence and transmigration through TNF-stimulated endothelium, and induce neutrophil degranulation. The degranulation of neutrophils subsequently releases reactive oxygen species and lytic enzymes such as elastase, which damage endothelial cells and tissues. This phenomenon is suggested to be responsible for the extensive damage to vessel walls that is seen in WG/GPA.[30]

The alternative complement pathway has also been shown to be involved in this process, and C3 complement deposition is found in more than half of tissue biopsies in patients with AAV.[31,32] *In vitro*, activation of primed neutrophils by ANCA leads to the release of factor B as well as factor C3. This further causes the activation of the complement pathways that release the split product C5a, which functions as a strong chemotatic factor for neutrophils, but is also able to prime neutrophils for interaction with ANCA. This process thus forms a perpetual cycle for neutrophil recruitment and activation, leading to ongoing vascular inflammation and tissue damage.[33]

The granulomatous inflammation observed in different tissues in WG/GPA suggests a strong role for the T-cell response, and both Th17 cells and regulatory T (Treg) cells are now receiving attention as potential players in disease development.[28,34] Other T-cell subtypes currently being investigated in the pathogenesis of AAV include $CD8^+CD57^+$ and natural killler-like CD4 cells, but their role has not yet been clearly defined.[35,36] The role of B lymphocytes and memory plasma cells in immune regulation, antibody production and pathogenesis has now been well established in autoimmune disease,[37,38] and plasma B cells have been demonstrated to have a role in the production of autoantibodies, including ANCA.[39,40] Activated peripheral B cells have also been

linked to disease activity and severity in WG/GPA,[41] and treatment regimens targeting B cells have thus been advocated in AAV (Figure 2).[42,43]

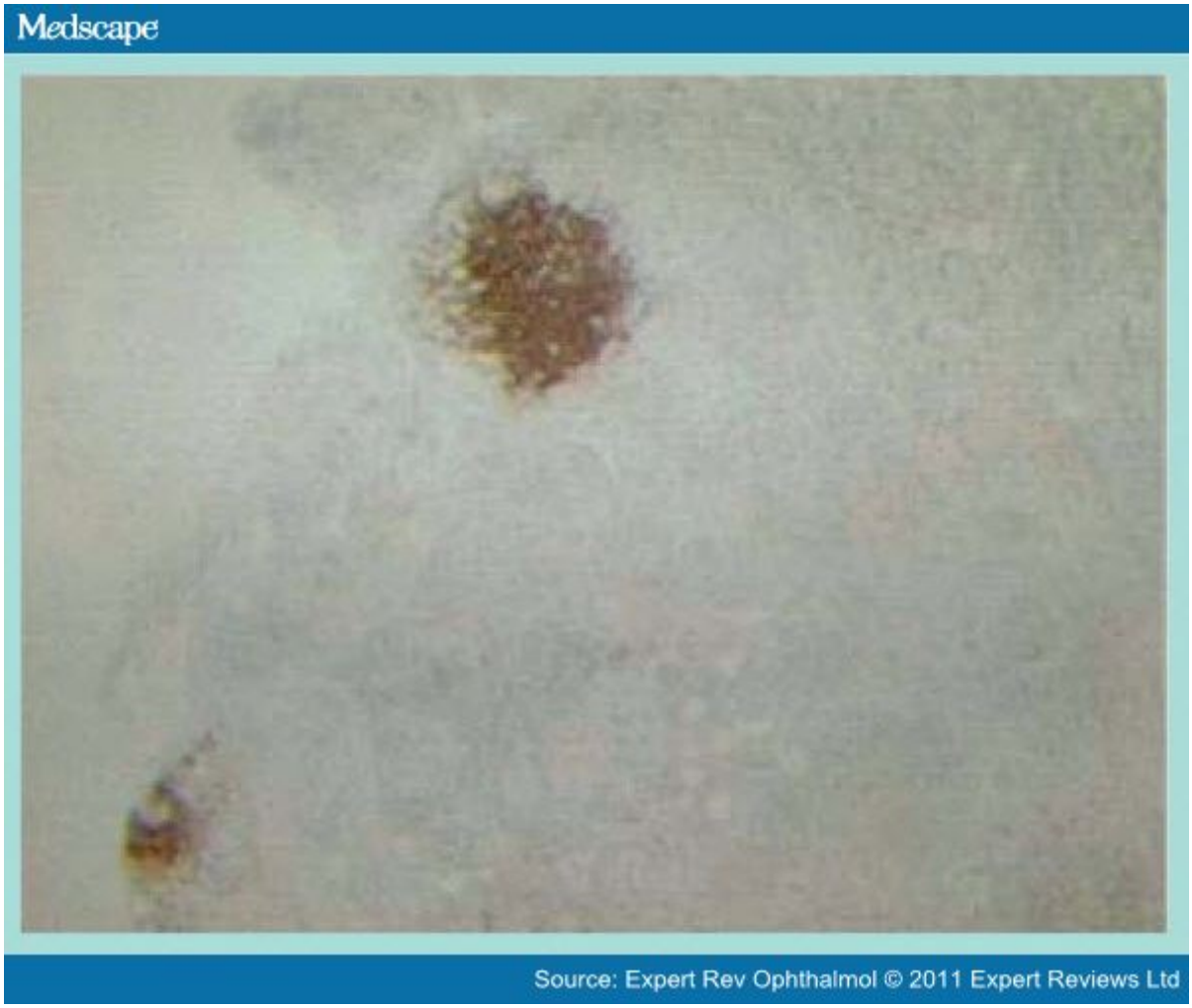

Figure 2.

Wegener's granulomatosis/granulomatosis with polyangiitis biopsies indicating the presence of $CD20^+$ B cells (stained brown).

Staphylococcus aureus has been implicated in the pathogenesis of AAV, as low-grade chronic infection in the upper airway can cause release of proinflammatory cytokines that can locally prime neutrophils that are then further activated by ANCA.[44] In addition, patients who are PR3-ANCA-positive have antibodies reactive with a protein produced from PR3 antisense RNA, and have been shown to have an amino acid sequence that has homology with proteins from many microbes and viruses, implicating molecular mimicry in the pathogenesis.[45] Recently, anti-human lysosomal membrane-associated protein (LAMP) 2 antibodies have been found to be present in 100% of AAV patients with active renal involvement, and in 93% of a cohort of 84 patients with either WG/GPA, MPA or renal-limited vasculitis.[46] Interestingly, anti-LAMP2 antibodies have also been shown to target the adhesion elements FimH (fimbriae) on Gram-negative bacteria, thus suggesting a link

between infection and vasculitis through molecular mimicry.[47] However, there has been no general consensus on this finding, and further confirmatory studies are required.

Diagnosis

The diagnosis of WG/GPA, particularly the limited form, requires a heightened level of clinical suspicion based on clinical presentation and pathological criteria, particularly as the severity of disease in WG/GPA may range from a nonspecific inflammatory picture involving only one site or organ, to fulminant multiorgan vasculitis that can lead to death.[47]

In systemic WG/GPA, c-ANCA were found to be positive in 74% of patients, with anti-PR3 detected in 87%.[48,49] One study conducted in Norway quoted a diagnostic sensitivity of 70% for c-ANCA and 63% for PR3-ANCA, with a specificity of 97 and 99%, respectively, for the diagnosis of WG (27 out of 319 patients).[50] In current clinical practice, ANCA detection is used for the diagnosis of WG/GPA and as an indicator of the disease activity. However, the ANCA titer is not entirely reliable in monitoring disease activity, and should not be solely relied upon in the management of WG/GPA patients.[51]

The ACR and The International Consensus Conference at Chapel Hill (CHCC) have proposed classification criteria and definitions, respectively, for the purpose of epidemiological studies for AAV, including WG/GPA. However, these classifications are rather limited, particularly as they did not include the presence of ANCA as a diagnostic criterion.[52] In 2007, a stepwise algorithm was developed by consensus between a group of doctors interested in the epidemiology of vasculitis, for the classification of AAV and polyarteritis nodosa to address the lack of compatibility between ACR and CHCC.[53] This includes a four-step algorithm incorporating both the ACR and CHCC systems that has been shown to be effective in classifying patients into a single category.[54,55] Nevertheless, all three of the aforementioned classification criteria have been developed mainly for clinical studies and have their own individual limitations,[56,57] and thus should not serve as a definitive tool for clinical diagnosis.[58]

In pediatrics, a EULAR/Pediatric Rheumatology European Society (EULAR/PReS) working group has recently developed classification criteria for childhood vasculitis using a Delphi technique.[59] Agreement was reached to classify childhood vasculitis according to vessel size, with small-vessel diseases subdivided into granulomatous and nongranulomatous. The ACR criteria for WG/GPA were modified to include two new criteria: the presence of subglottic, tracheal or endobronchial stenosis; and the presence of high levels of PR3-ANCA or positive cANCA by indirect immunofluorescence.[58]

In the limited form of WG/GPA, like ocular WG/GPA, classical features of the disease are often lacking and may not fit any classification or definition. In addition, in the limited form of WG/GPA, ANCA presence and level is a poor indicator for diagnosis and disease activity, as ANCA is only found to be positive in 50–65% of patients.[21,60] As a result, the diagnosis of limited WG/GPA may be missed or delayed and only detected once disease progresses to a more severe systemic form.[10,47]

Ophthalmic Features of WG/GPA

Ocular involvement occurs in 50–60% of patients with WG/GPA,[61] and it can affect any structure of the eye, from the eyelid and orbit to the optic nerve. Ocular WG/GPA can either manifest *de novo*, as disease spread from contiguous structures such as the sinuses, or as part of systemic WG/GPA.

Ophthalmic presentations vary from an orbital mass formation (orbital granuloma), ocular vasculitic changes, adnexal inflammation and nasolacrimal duct changes.

Ocular inflammation can occur with or without systemic manifestations of WG/GPA,[62] and both limited and systemic forms of the disease can result in severe ocular morbidity, with visual loss occurring in up to 8% of patients. In 8–16% of patients, ocular manifestations may be the initial presentation of WG/GPA, but diagnosis is often difficult as the signs and symptoms are usually nonspecific and tend to overlap with those of other orbital inflammatory disorders.[4,63] Patients with ocular WG/GPA have been reported to progress to the systemic form by one study,[64] but others have failed to observe the same pattern.[65,66] Only 50–65% of the limited form of WG/GPA is found to be ANCA positive.[67–69]

Scleritis & Episcleritis

Scleritis is inflammation involving the entire thickness of the sclera. Scleritis of the necrotizing type is one of the common ophthalmic manifestations of WG/GPA, occurring in 50% of patients (Figure 3).[70,71] It may be the initial clinical presentation of WG/GPA,[72] and typically presents with severe deep boring pain that may radiate to the temple and jaw, and red, tender eyes. The pain typically worsens at night, waking the patient up from sleep. Areas of severe vasculitis causing capillary closure in the deep episcleral vascular bed are characteristic of this disease and are seen as areas of capillary nonperfusion on ocular examination. This can lead to infarction and necrosis of the involved sclera, exposing the underlying uvea.[67] Despite thinning of the sclera, ocular perforation is rare. During inflammation, adjacent ocular structures such as the cornea, trabecular meshwork and ciliary body may also be involved and can lead to complications such as keratitis, corneal ulceration, uveitis, ocular hypertension or glaucoma.[68]

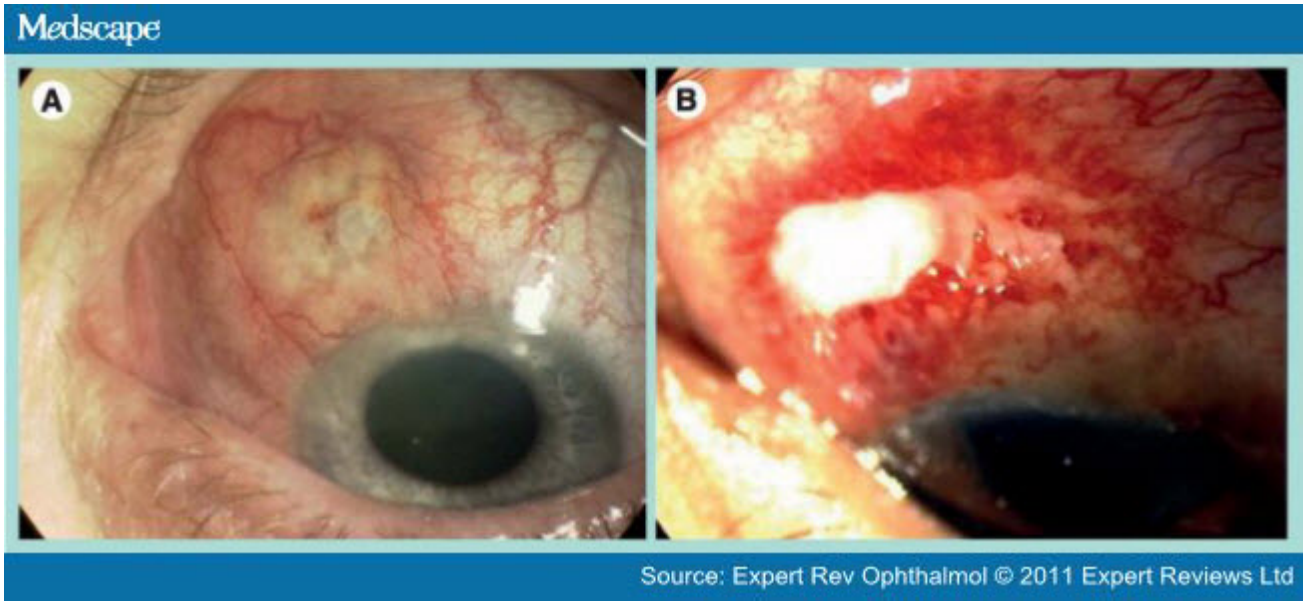

Figure 3.

Clinical appearances of necrotizing scleritis. In both **(A)** and **(B)**, the necrotic area is seen as a white patch on examination due to capillary closure of the deep episcleral vascular beds and deep sclera vascular plexus that overlies the sclera. Disease is more marked in **(B)**.

Episcleritis is characterized as inflammation of the loose episcleral tissues between the conjunctiva and sclera.[4] Although episcleritis is not usually associated with systemic diseases it has been described in patients with WG/GPA.[69] It usually has a less-severe presentation, where patients may complain of red eye with mild discomfort and epiphora. The eye is seen to be either diffusely or locally injected on examination. Episcleritis typically runs a milder course compared with scleritis and is generally self-limiting with few or no ocular complications. However, in WG/GPA it has been reported that patients with episcleritis often require a short course of topical glucocorticoids.[21]

Keratitis

Peripheral ulcerative keratitis (PUK) is also a common ocular manifestation of WG/GPA (Figure 4),[70] and bilateral PUK has been reported in the past.[73] In this situation, it is often associated with scleritis, and ocular pain may be pronounced. Other symptoms may include tearing, photophobia and reduced vision. Corneal stromal infiltration and clouding, with invading vessels from the limbus, are typically seen. Breakdown of the overlying corneal epithelium resulting in ulceration and stromal thinning can occur, and if left untreated, may result in corneal perforation.[4] In WG/GPA, corneal melt may occur earlier in the disease course, which can progress quickly, resulting in visual loss.[67,79]

Figure 4.

Peripheral ulcerative keratitis evidenced by guttering of the peripheral superior cornea.

Orbit

The orbit is another common ocular structure involved in WG/GPA. The majority of patients with WG/GPA and orbital involvement have contiguous disease involving the nose and paranasal sinuses, although orbital WG/GPA may also occur *de novo* with orbital granuloma formation or orbital vasculitis. Common presentations of orbital WG/GPA include proptosis due to an orbital mass, diplopia, periorbital swelling, reduced vision, eye redness and pain and epiphora.[4,21] Patients with orbital WG/GPA are prone to develop significant visual loss and ocular complications, with 20–50% of patients developing severe visual loss (i.e., six out of 60 or less).[75]

Proptosis is the most common ocular presentation of orbital WG/GPA (Figure 5).[21] It is an important sign, as together with respiratory disease or glomerulonehphritis, highly suggests the diagnosis of WG/GPA.[10] Patients with proptosis and lid destruction are prone to develop exposure keratopathy, which can lead to corneal ulceration, ocular perforation and blindness.[4] Orbital myositis in WG/GPA may occur as a result of inflammation of the extraocular muscles or vasculitis of the vasa nervorum, and patients mainly present with symptoms of diplopia and pain on ocular movement and signs of restriction of ocular motility.[76]

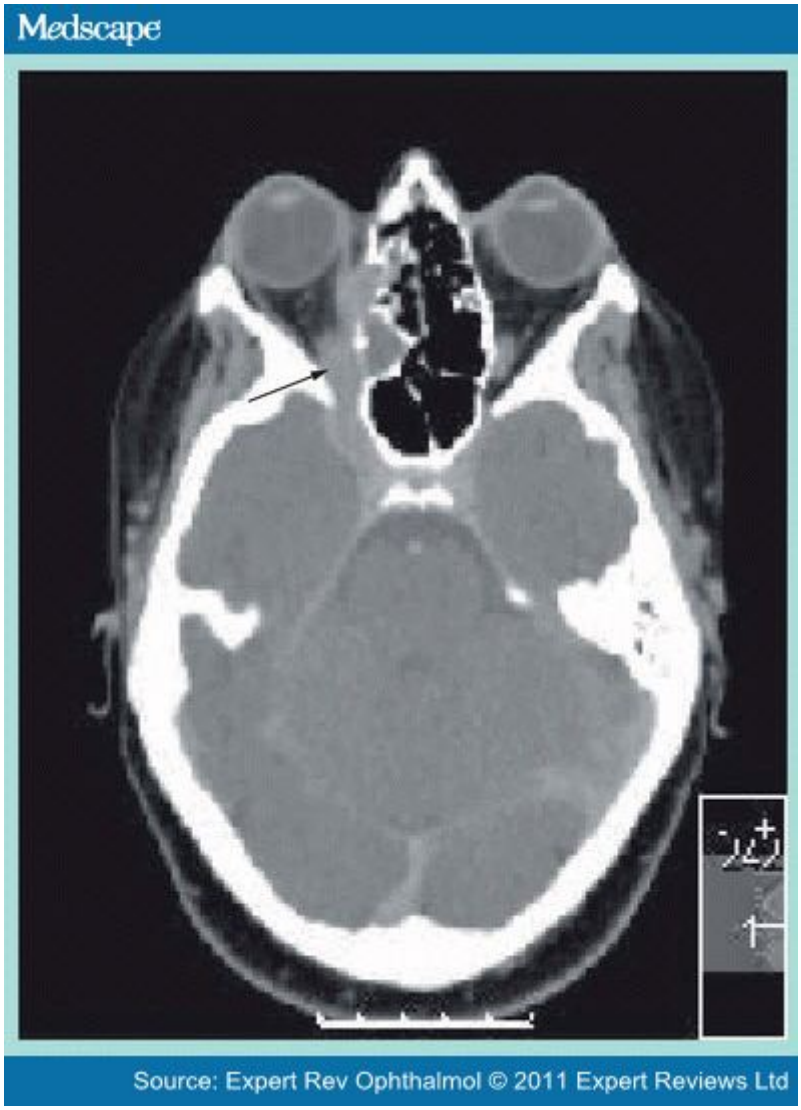

Figure 5.

Orbital Wegener's granulomatosis/granulomatosis with polyangiitis with an orbital mass (arrowed) and subtle right eye proptosis seen on computed tomography scan. The orbital granuloma can be seen to extend through the superior orbital fissure and into the cavernous sinus.

Orbital apex infiltration of WG/GPA may involve the optic nerve resulting in painless optic neuropathy, optic nerve swelling and ensuing atrophy; further infiltration may cause painful ophthalmoplegia and blindness.[77] Ophthalmoplegia may also be a result of vasculitis involving the cranial nerves supplying the extraocular muscles.[4] Orbital contracture syndrome can occur as a complication of orbital WG/GPA and is described as proptosis followed by development of enophthalmos with radiographic evidence of fibrotic changes in the orbit. This complication is thought to be due to the proliferation of fibrous tissue replacing areas of acute inflammation and necrosis where ensuing contracture leads to restriction of motility and enophthalmos. The optic nerve may also be involved in this, causing further visual loss. As with other fibrosing manifestations of WG/GPA, socket contracture responds poorly to immunosuppressive therapy.[78]

Eyelid, Adnexal & Conjunctiva

Eyelid involvement in WG/GPA is uncommon, but includes lid granulomas and ptosis. In severe cases, lid destruction may also occur. The 'yellow-lid sign', when associated with orbital inflammation, has been suggested to point to the clinical diagnosis of WG/GPA,[79] and is described as resembling florid xanthelasma despite the patient having normal lipid metabolism.[80] Dacroadenitis and lacrimal gland enlargements are recognized features of adnexal involvement in WG/GPA and may cause ocular sicca syndrome.[81] Dacrocystitis and epiphora can occur as a consequence of nasolacrimal disease, and nasolacrimal blockage in WG/GPA may be a result of inflammatory spread from adjacent sino–nasal inflammation, or a direct consequence of focal WG/GPA inflammation.[4,21] Conjunctival involvement occurs in 16% of patients with WG/GPA,[82] manifesting as ulcerative and necrotic conjunctivitis, which may result in marked cicatricial changes of the conjunctiva.[21]

Uveitis, Retina & Choroid

Uveitis is a rare manifestation in WG/GPA and is seen only to occur in 3% of patients according to one study.[75] Often, uveitis in association with WG/GPA occurs concurrently with scleritis and is termed sclerouveitis, and probably carries a poorer ocular prognosis.[4]Retinal and choroid involvement in WG/GPA is also rare, but recognized retinal manifestations of WG/GPA include retinitis, chorioretinitis, macular edema, exudative retinal detachment and retinal necrosis.[4] Retinal vein occlusion has been reported, and is thought to occur owing to external wall compression by tissue granulomas.[83] By contrast, central retinal artery occlusion, which can occur bilaterally and simultaneously in WG/GPA, is associated with vasculitis; prompt treatment of the inflammation may improve the visual prognosis in these patients.[84–86] Other rare presentations of WG/GPA in the retina and choroid include sclero–choroidal granulomas mimicking uveal melanoma,[87] vitreous hemorrhage from chorioretinal and ciliary body granulomas [88] and acute multifocal placoid pigment epitheliopathy.[89]

Systemic Clinical Features of WG/GPA

Wegener's granulomatosis/granulomatosis with polyangiitis can affect any organ system, although the disease has a tendency to affect the upper and lower respiratory tract and kidneys. However,

the clinical presentation may be nonspecific, with just symptoms of fever, malaise, anorexia, weight loss and arthralgia.[90]

In a study conducted by Lane *et al.* in 2005, the ear, nose and throat was shown to constitute the most common initial symptom (81%) in WG/GPA. Ear, nose and throat also commonly makes up the first hospital speciality that reviews patients with WG/GPA.[91] The upper and lower respiratory tract systems are commonly affected in WG/GPA, occurring in up to 85% of patients.[10,92] Chronic sinusitis that is unresponsive to treatment is the most frequent initial presentation, occurring in approximately 70% of patients with WG/GPA. Subglottic stenosis is a life-threatening complication of WG/GPA and should be suspected in a WG/GPA patient who presents with dyspnoea in the absence of active pulmonary disease. Pulmonary involvement occurs in approximately 67% of WG/GPA patients, although 85% eventually develop lung disease.[91,92]

Renal involvement occurs in 75–80% of patients with WG/GPA, although only 20% of patients will have features of active glomerulonephritis.[16] Once the renal system is involved, the disease may accelerate rapidly despite the patient being asymptomatic. Prognosis is related to serum creatinine at presentation.[55] Rapidly progressive glomerulonephritis is the most severe renal manifestation of WG/GPA and, if untreated, can lead to the need for dialysis or transplantation within weeks. The pulmonary renal syndrome is a combination of diffuse alveolar hemorrhage and rapidly progressive glomerulonephritis. It is a rare but serious complication of various systemic disorders, with the majority of cases related to AAV.[56]

Approximately two-thirds of patients with WG/GPA complain of musculoskeletal symptoms. WG/GPA involves the nervous system in 20–50% of patients, most frequently affecting the peripheral nervous system, with mononeuritis multiplex present in some 22%. Gastrointestinal manifestations of WG/GPA are uncommon but are now increasingly recognized as part of this disease. Lesions are seen when performing colonoscopy or enteroscopy, manifesting as multiple intestinal ulcerations that can involve both the large and small bowels,[93,94] and can lead to multiple intestinal perforations.[93,95] Vasculitis of the coronary vessels in WG/GPA may lead to myocardial ischemia.[96] It has been reported in one study that WG/GPA patients seem to have an increased morbidity from ischemic heart disease compared with the rest of the population.[97] There is also an apparent increased risk of first WG/GPA relapse after initial remission in patients with cardiovascular manifestations.[98]

Histology in Ocular WG/GPA

The histopathology of WG/GPA, based on postmortem examination of WG/GPA patients, consists of a variable mixture of granulomatous lesions with fibrinoid necrosis or microabcesses, tissue necrosis and vasculitis of small- to medium-sized vessels. Granulomas are said to occur early in the disease process, with vasculitis developing in the later stages.[99] In 2008, Ahmed *et al.* described the pathologic features of biopsies from patients with ophthalmic WG/GPA, and reported that WG/GPA was associated with granulomatous foci, collagen necrosis, neutrophil/nuclear dust, plasma cells and infiltrating eosinophils.[100] The presence of granular degeneration of the interstitial collagen, mummification of the collagen with disappearance of fibroblastic nuclei, and a polymorphous infiltrate exhibiting plasma cells, lymphocytes, neutrophils and eosinophils within the epithelioid granulomas were said to be particularly suggestive of the diagnosis of WG/GPA.[98] By contrast, granulomatous inflammation without vasculitis is more characteristic of other orbital inflammatory diseases.[101]

Following the introduction of rituximab (RTX; an anti-CD20 antibody), there has been a growing interest in the potential role of B cells in the pathogenesis of WG/GPA. However, B-cell infiltrates in WG/GPA are not found in all involved organs and T cells and macrophages predominate in tissue biopsies. The presence of eosinophils has also been demonstrated in biopsies.[64] Interestingly, nuclear debris has been repeatedly described in pathological reports in WG/GPA,[60,64,100] but does not seem to be seen in any other ocular inflammatory conditions.

Classic histological features of WG/GPA are not always present in biopsy material. This is possibly more so in the case of the limited form of WG/GPA, especially ocular WG/GPA, where biopsy specimens are often too small to capture the whole pathologic picture, or the inflammatory process is too mild to mount a typical tissue cellular response.[102]

Orbital Imaging

Radiographic investigations are used frequently in the investigation of orbital masses. Imaging with computed tomography (CT) and/or MRI is of value in the diagnosis of orbital WG/GPA to demonstrate orbital mass lesions and delineate involvement of adjacent structures, and radiological features of orbital and sinus involvement are detectable in approximately 70% of patients with clinical orbital involvement.[103–105]

CT Scan

Computed tomography scanning is more effective at evaluating sinus opacification and bone invasion, which is a common finding in WG/GPA.[106] Classically, appearances on CT are of an ill-defined soft tissue mass that may cloak the globe, obscuring the optic nerve and extraocular muscles, with associated local bony destruction. On unenhanced CT images, orbital lesions appear homogeneous and isodense relative to the extraocular muscles. The contrast-enhanced CT appearance is that of a moderately heterogenous mass that was isodense or mildly hyperdense relative to nasal mucosa. Studies with contrast can show a wide range of enhancement characteristics with extreme diversity of enhancement values.[107] There are no radiological features that can make a specific diagnosis of WG/GPA.

MRI

Magnetic resonance imaging may be helpful in characterizing orbital lesions in WG/GPA. On T1- and T2-weighted images, low-to-intermediate signal granulomas can be distinguished from the surrounding orbital fat tissues and extraocular muscles.[108,109] However, granulomas can only be depicted as low-signal-intensity lesions on MRI in the later stage of granulomatous transformation and, in the initial inflammatory process of WG/GPA, it is not possible to differentiate between mucosal inflammation and granulomatous tissue on MRI. A marked decrease in T2 signal is a characteristic of WG/GPA, but similar findings can occasionally be seen with idiopathic orbital inflammation, chronic lacrimal gland sarcoidosis and in metastatic melanoma of the orbit.[107,110]

Following contrast, varying degrees of enhancement may be observed.[109] After gadolinium injection, granulomas may show homogenous [104] or heterogeneous signal enhancement, or, in a minority of cases, no enhancement.[108] Therefore, Wegener's granulomatosis should be included in the differential diagnosis of patients with low-signal-intensity lesions on T1- and T2-weighted SE sequences of the nasal cavity, paranasal sinuses and orbits. The unenhanced, nonfat-suppressed T1-weighted sequence is the preferred method for lesion detection in orbital WG/GPA.[110] Bony erosion of the orbit and sinuses has also been demonstrated in WG/GPA and most often affects the ethmoid sinuses, nasal septum and medial walls of the orbit.[108,109]

Differential Diagnosis of Ocular WG/GPA

The overlapping clinical presentation of orbital WG/GPA with other orbital inflammatory conditions poses difficulty in establishing a definitive diagnosis. The differential diagnosis of orbital inflammatory disorders can be broadly divided into infectious, inflammatory and neoplastic causes. Thyroid eye disease is the most common cause of systemic-related orbital inflammation and may be unilateral or bilateral.[111] Typical features of ocular thyroid eye disease, such as lid lag and lid retraction, together with systemic symptoms of thyroid dysfunction, may help in making the diagnosis. Abnormal thyroid function tests and raised anti-thyroid antibodies may also assist in the diagnosis, although normal levels do not exclude it.

Orbital cellulitis from bacterial or fungal infections must be excluded prior to commencement of immunosuppressive treatment, particularly when there is a history of trauma to the sinuses or dental infection or intervention.[21] Patients with orbital cellulitis often present with symptoms of infection such as fever, tachycardia, leukocytosis and discomfort, and blood cultures may be useful in identifying the infective agent.

IgG4-related disease is a systemic lymphoproliferative disorder where affected organs show hyper-IgG4-γ-globulinemia and IgG4-producing plasma cell expansion with fibrotic or sclerotic changes. In the orbit it commonly affects the lacrimal gland where patients present with bilateral symmetrical lacrimal gland swelling and dacryoadenitis. Other ocular symptoms include eyelid or periocular swelling and proptosis. The diagnosis of IgG4-related disease is confirmed with serologically high levels of serum IgG4 (>135 mg/l) and marked IgG4-positive plasmacyte infiltration (>40% IgG4-positive/IgG-positive cells in five high-power fields) into lacrimal biopsy tissues.[112,113]

Apart from WG/GPA, other inflammatory disorders with orbital presentations include sarcoidosis, idiopathic orbital inflammation, Churg–Strauss syndrome, Erdheim–Chester syndrome and Tolosa–Hunt syndrome. Rheumatoid arthritis may present with scleritis, episcleritis and PUK. Neoplastic disorders that may present with proptosis include rhabdomyosarcoma and lymphoma. Tumor metastasis to the orbit from a remote primary tumor should also be considered.[114,115]

Treatment

Wegener's granulomatosis/granulomatosis with polyangiitis is a fatal disease if left untreated, and the natural history is for only 50% of patients to survive 5 months after diagnosis and for 80% to die within 1 year, mainly from renal disease [10] and secondary infection. Treatment with corticosteroids (CSs) alone only moderately increased the mean survival time to 12 months, but the present combination of CS with other immunosuppressive drugs such as cyclophosphamide (CYC) has been shown to achieve disease remission in 93% of patients [116] and improve survival.

The treatment of ocular involvement in WG/GPA largely depends on the clinical presentation, severity of the disease and whether there are extraocular organ involvements. There are very few clinical trials that specifically address the treatment for ocular WG/GPA. Many reports on the subject are mainly based on single case reports and case series. Frequently, the management of ocular WG/GPA requires treating the underlying systemic disease with CSs and cytotoxic agents; thus, treatment decisions can be guided by clinical trials for systemic WG/GPA.

Although clinical presentation may be confined to only ocular manifestations of WG/GPA, the disease may progress to involve other organs over time and, once WG/GPA is suspected, collaboration between ophthalmologists and physicians for thorough evaluation, investigation, management and future monitoring is essential. Identification of eye and extraocular disease of

WG/GPA will require systemic therapy. In cases where evidence of WG/GPA is strong, such as bilateral ocular inflammation, necrotizing scleritis, particularly with corneal infiltrates, or eye symptoms with corresponding sinus or upper respiratory characteristics of WG/GPA, treatment should be advocated even though serology testing for ANCA is negative.

Topical treatments with NSAIDs or CSs are only used in the treatment of episcleritis or mild anterior uveitis, as ocular WG/GPA tends not to respond to topical agents. Keratitis in WG/GPA will require treating the underlying systemic condition, and in cases of impending perforation, adhesive glue and tectonic grafting may be necessary.[71] In cases of isolated non-necrotizing anterior scleritis, the use of oral NSAIDs or oral CSs may be advocated. Local injection of CSs may be considered for rare cases of unilateral uveitis and refractory nonnecrotizing scleritis with no evidence of active systemic disease.[117]

Aggressive immunosuppressive treatment with CYC and CSs (1 mg/kg/day) is necessary for the more severe cases of ocular WG/GPA, such as necrotizing scleritis, bilateral ocular involvement, posterior scleritis, orbital or adnexal presentation and refractory cases. Holle*et al.* reported that the most frequent indication for CYC in localized WG/GPA is patients with orbital masses and cavitating pulmonary masses.[66] Recently, the management of WG/GPA has been moving away from CYC owing to its well-recognized serious adverse effects and the emergence of new treatment regimens with biologics such as RTX, which is recently gaining popularity as the first line of treatment in the management of WG/GPA.

Cyclophosphamide, when considered for treatment in WG/GPA, may be given orally or intravenously as pulses, initially at a 2 week interval, then three-times weekly with oral CS (1 mg/kg/day). Intravenous pulse CYC has been shown to have a similar remission rate to continuous oral CYC and is advocated to reduce the toxicity of the drug (CYCLOPS study),[118] but may have a higher relapse rate. With this combination, remission is seen in 70–90% of patients with systemic WG/GPA, with mortality rates of 9% at 18 months.[119]However, 30–70% of patients relapse, and up to 42% experience severe side effects from the treatment. CYC is known to cause many serious adverse effects such as serious infection, bladder cancer and increased malignancy risk (two- to four-fold), as well as other complications, including amenorrhea, infertility, cystitis, hematuria and transient alopecia.[47,120]

Rituximab has been reported to be effective in treating various ocular manifestations of WG/GPA, such as PUK,[121] relapsing necrotizing scleritis,[43] optic nerve infiltration [76] and optic neuritis,[122] although remission may take 7 months to occur.[123] RTX has also been found to be effective in inducing remission in refractory ophthalmic WG/GPA, and is capable of inducing extended remission compared with other biologics and conventional treatments.[124] RTX is a chimeric monoclonal anti-CD20 B cell antibody that depletes B cells. It is now being more frequently used as a treatment regimen for WG/GPA instead of the standard CYC induction protocol recommended by the European Vasculitis Study Group (EUVAS). RTX has been reported to be as effective in the treatment of AAV as CYC in two clinical trials.[40,125,126]

The RTX versus CYC for ANCA-Associated Vasculitis (RAVE) study in 2010 was a head-to-head, multicenter randomized double-blind noninferiority trial, comparing CS plus RTX (375 mg per square meter of bodysurface area per week for 4 weeks) with CS plus CYC (2 mg per kilogram of bodyweight per day) for remission induction in 197 patients with severe ANCA positive-associated vasculitis.[125] In both arms, CSs were tapered off over a period of time in the usual regimen. The primary end point of the study was remission of disease without the use of prednisolone at 6 months, and RTX was found to be noninferior to CYC in achieving this. Interestingly, in inducing

remission in relapse diseases, RTX was found to be more effective than CYC. Surprisingly, there was no difference in the adverse event rate between the two groups.

Similarly, the RTX versus CYC in ANCA-Associated Renal Vasculitis (RITUXVAS) trial compared a standard CS regimen plus either RTX with two intravenous CYC pulses or intravenous CYC for 3–6 months followed by maintenance azathioprine (AZA).[126] The primary end point of the study was remission at 12 months, and this was equal in both groups. There were no significant differences in severe adverse events, and renal function improved in both groups. The result shows that RTX can spare CYC in the treatment of AAV, thus reducing the exposure of patients to CYC.

These two studies not only demonstrate that RTX is an effective treatment for AAV, but it also appears to be well tolerated by patients. However, being a relatively new treatment, length of disease remission, disease relapse rate and its long-term safety profile is as yet uncertain; late-onset neutropenia in patients treated with RTX for rheumatic diseases has recently been reported, the highest incidence being seen in WG/GPA and systemic lupus erythematosus.[122]

Apart from CYC and RTX, in limited WG/GPA it has also been suggested that the combination of methotrexate (MTX) and CS achieves remission rates of approximately 70%.[127] In patients with early systemic WG/GPA (defined by EUVAS as AAV with systemic vasculitis without organ-threatening or life-threatening disease), the combination of MTX (20–25 mg per week) with CS (1 mg/kg/day and gradually tapered) has been recommended by EULAR as a less-toxic alternative to CYC for inducing remission (NORAM study).[106,128]However, MTX was found to be less effective in achieving remission in patients with extensive disease and pulmonary involvement compared with CYC, and had a higher relapse rate in this group. Nevertheless, MTX is widely considered to be the drug of choice for patients with early systemic WG/GPA.

Adjunctive Anti-TNF-α Agents

The anti-TNF-α agents infliximab and etanercept have been extensively studied for their potential role as remission-inducing therapies for vasculitic diseases, although not for ocular WG/GPA *per se*.

Infliximab has been reported to be effective in treating cases of WG/GPA with ocular involvement in several isolated reports.[129,130] In a previous study, infliximab, a chimeric monoclonal antibody directed against TNF-α, was shown to be effective at inducing remission in WG/GPA,[131,132] but the numbers representing ocular WG/GPA were very small (three out of 12 patients). However, recently, a cohort study by Morgan *et al.* showed that the addition of infliximab to standard therapy was found not to influence remission rates, adverse events, damage index scores, relapse rates or biomarker levels; thus, infliximab appears not to have further clinical benefits for patients with active AAV.[133]

Importantly and unexpectedly, the WGET, which included 30% of patients (n = 180) with eye involvement, found that etanercept was ineffective for the maintenance of remission in patients with Wegener's granulomatosis and was associated with a high rate of treatment-related complications, including solid-organ tumors.[134] A recent report of the long-term follow-up of these patients revealed that compared with the general population, the risk of solid malignancies in the etanercept group was increased.[135]

Maintenance Therapy

Maintenance therapy following disease remission is recommended in AAV patients owing to the high tendency for disease relapse to occur. It has been suggested that remission therapy should be

continued for at least 18 months, particularly in WG/GPA.[128] In cases of ocular WG/GPA, this practice would also seem beneficial owing to the potential aggressive and destructive nature of this disease, although it is uncertain whether such a long maintenance period is necessary, and what treatment regime is best for remission maintenance in patients with ocular WG/GPA. MTX and AZA have both been shown to be equally as effective as CYC in maintaining disease remission in WG/GPA, and the CYCAZAREM trial showed that, in patients with generalized vasculitis, the withdrawal of CYC and substitution of AZA after remission did not increase the rate of relapse, and thus the duration of exposure to CYC may be safely reduced.[136]

Mycophenolate mofetil (MMF) has also been demonstrated to be effective in sustaining short- and medium-term remission and is a well-tolerated drug.[137] Surprisingly, however, in the IMPROVE study, MMF was found to be inferior to AZA in maintaining disease remission among AAV patients.[138] MTX has also been recommended by EULAR as an effective remission maintenance therapy.[128] Other options include leflunomide, a pyrimidine synthesis inhibitor that targets T cells by inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase, thus limiting pyrimidine synthesis. This has also been reported to be an effective alternative immunosuppressive drug for remission maintenance in WG/GPA,[139,140] although it has been associated with increased frequency of adverse events.[140]Unfortunately, relapse rates remain high,[141] particularly in patients who are still ANCA positive at time of remission, and it is therefore recommended that treatment is maintained for up to 18 months post remission, and continued close follow-up of patients during this period is mandatory.

Bacterial and viral respiratory tract infections have been postulated to trigger relapses in patients with PR3-positive vasculitis. Data have suggested that treatment with cotrimoxazole may be beneficial, because this antibiotic could act by eliminating the offending microbe and thereby stopping the initiating stimulus in these patients. Treatment with cotrimoxazole has been shown to reduce the incidence of relapses in patients with WG/GPA in remission in previous studies,[142,143] and should be considered in cases of persistent endonasal activity of WG/GPA, together with *S. aureus* carriage.[144] Cotrimoxazole was also recommended by both EULAR [128] and the British Society for Rheumatology (BSR) and British Health Professionals in Rheumatology (BPHR)guidelines [145] as prophylaxis against*Pneumocystis jerovicii* infection in patients with WG/GPA.

Surgical Intervention for Orbital WG/GPA

Surgical procedures in the management of ocular WG/GPA are largely performed for diagnostic purposes; that is, orbital biopsy. In cases of nasolacrimal duct obstruction, dacryocystorhinostomy is shown to be effective in restoring nasolacrimal duct function affected by WG/GPA. Outcomes of dacryocystorhinostomy are mostly best when surgery is performed during disease remission and low ANCA titers. As mentioned previously, in cases of severe ulcerative keratitis with impending ocular perforation, using adhesive glue and performing tectonic corneal grafting is necessary.

Severe ocular proptosis secondary to WG/GPA is often unresponsive to conventional medical therapy. In severe cases of proptosis associated with pain and optic nerve compression that are refractory to medical therapy, surgical orbital decompression should be considered.[146] Prompt diagnosis and surgical decompression following acute visual deterioration can lead to good visual outcome.[147] However, related ocular symptoms such as diplopia may still be difficult to resolve, even when orbital decompression is performed. Other indications for surgery in ocular WG/GPA are cataract surgery and trabeculectomy for the control of glaucoma, both common complications in WG/GPA developed either from ocular inflammation or steroid induced.

Treatment of Systemic WG/GPA

In 2007, the EUVAS devised a system of disease subgroupings for AAVs, based on the severity of WG/GPA presentation, to guide therapy ( ).[145] Current treatment for these diseases is based on assessing the patient's clinical severity and disease extent.

9.1.1.11 Table 1. Disease categorization of antineutrophil cytoplasm antibody-associated vasculitis (based on the European Vasculitis Study Group classification).

Category	Definition
Localized	Upper and/or lower respiratory tract disease without any other systemic involvement or constitutional symptoms
Early systemic	Any, without organ- or life-threatening disease
Generalized	Renal or other organ-threatening disease, serum creatinine <500 µmol/l (5.6 mg/dl)
Severe	Renal or other vital organ failure, serum creatinine >500 µmol/l (5.6 g/dl)
Refractory	Progressive disease unresponsive to glucocorticoids and cyclophosphamide

Data taken from [19,20].

As with ocular WG/GPA, treatment for systemic WG/GPA is focused on remission induction and thereafter maintenance therapy. Treatments for early systemic and generalized disease are similar to the treatment regime as previously described. As for severe WG/GPA, additional treatment regimens have also been advocated, such as CYC (intravenous or pulse) and steroid with adjuvant plasma exchange (plasmapheresis).[148,149] Intravenous immunoglobulin has been shown to reduce disease activity in refractory AAV, although the effect was not maintained beyond 3 months.[150]

Other Treatment Regimens

15-deoxyspergualin has been used with some success in refractory WG/GPA cases,[151] and is a synthetic derivative of spergualin, a protein from *Bacillus laterosporus* that is capable of preventing T-cell and B-cell maturation. It may offer a safer alternative to CYC for induction therapy, but is not yet supported for routine clinical use. Anti-T-cell antibodies, such as anti-thymocyte globulins, are polyclonal antibodies targeted against T-lymphocyte antigens that cause rapid depletion of T lymphocytes and have been investigated for use in refractory WG/GPA.[152] Anti-CD52 therapy (alemtuzumab; CAMPATH-1H) is a humanized monoclonal antibody to CD52 with anti-lymphocyte activity and has been shown to induce remission in AAV, although relapses and adverse events are common; its use in WG/GPA is considered experimental.[153]

Other treatment options for AAV on the horizon include hematopoietic stem cell transplant, intravenous high-dose AZA, intravenous immunoglobulins and abatacept.

Prognosis

Improved treatment regimens have resulted in improved survival and morbidity rates in patients with WG/GPA. Most studies now report a 5-year survival probability of 65–75% in these patients. The toxic profiles of these new drugs also appear to be more favorable and are better tolerated by patients compared with previous conventional standard treatment. Ocularly, significant visual problems are seen in up to 17% of cases, and in patients with orbital WG/GPA, significant visual loss occurs in 50% of patients. Visual losses in WG/GPA are largely a result of corneoscleral damage, macular edema, optic nerve compression from orbital mass or vascular occlusion. Prompt disease recognition, management and regular follow-up by an ophthalmologist is therefore mandatory.

Conclusion

Wegener's granulomatosis/granulomatosis with polyangiitis is a complex multisystem disorder with variable clinical presentation and disease manifestation. Despite better understanding of the disease process involved in WG/GPA currently, exact pathogenesis of the WG/GPA is still undetermined. There are no specific disease markers either in blood, histology or radiological investigations for WG/GPA, and this causes difficulty and delay in making the diagnosis. Ophthalmic involvement in WG/GPA is relatively common and diagnosis requires a heightened level of suspicion by ophthalmologists. As ocular signs may be the initial presentation of the disease, collaboration between ophthalmologists and physicians in the management of these patients is recommended, to optimize treatment and provide close monitoring in the hope of preventing progression and irreversible organ damage. Alternative drug treatment for WG/GPA with MTX and RTX with CS has been found to be equally effective as the conventional therapy of CYC and CS, and provides a potentially safer treatment option for patients.

Expert Commentary

The ocular manifestations of WG/GPA are relatively common, and there is increasing awareness among ophthalmologists of this disease. However, despite recent advances in the understanding of WG/GPA, it still remains a challenging disease to diagnose and manage. This is particularly more so for ocular WG/GPA, given its overlapping clinical presentations with other ocular inflammatory disorders, low percentage of ANCA positivity and the lack of classic histological findings in biopsies, thus making it indistinguishable from other orbital inflammatory diseases. To date, the pathogenesis of WG/GPA remains elusive and specific clinical signs or disease markers pathognomonic for WG/GPA are still absent. Successful treatment with anti-B-cell therapy in the form of RTX has indicated that B cells play a major role in disease development as well as T cells, but their precise interaction remains as yet undetermined.

Tissue damage remains an important problem with systemic vasculitis despite effective remission-inducing drugs. Only a fraction of patients with systemic vasculitis are unmarked by their disease. Unfortunately, there is no way of deciding which treatment regime would be best suited for individual patients. There is a healthy increase in the development of new treatment regimens for systemic vasculitic diseases that are targeting cell specific. However, recent studies looking into alternative and safer treatment options for the management of WG/GPA have shown some unexpected findings. One in particular was the comparison between the two purine inhibitors, MMF and AZA, for remission maintenance in WG/GPA. It was surprising that MMF, a newer generation

drug, was shown to be inferior to the older generation drug, AZA, in maintaining remission, where patients were found to relapse quicker on MMF and had a higher relapse rate.

Current studies and reports on RTX as a new treatment regime for WG/GPA have been encouraging. RTX currently appears to be an effective treatment for both the systemic and limited form of WG/GPA, and is well tolerated by patients. Although to date it appears to be a better treatment option when compared with CYC, recent studies have shown that the adverse events between the two treatment regimens were surprisingly comparable. Furthermore, as it is a new treatment method, the long-term safety profile of RTX is yet to be established.

Five-Year View

Newer biologic therapies will undoubtedly play a significant role in the management of WG/GPA in the future and, although the long-term safety profile of RTX remains uncertain, it holds a potential role as a substitute to CYC as the standard treatment for WG/GPA in the future. Further understanding of the immune response involved in vasculitides should result in the development of target-specific treatment regimens.

Identifying tissue markers or cellular patterns in tissues biopsies that are specific for WG/GPA would be of great value to aid and improve diagnosis, and the discovery of a key factor that could predict disease remission, disease extension or relapse would also be of great benefit. This could lead to a risk stratification and aid in the formulation of a rational treatment approach for WG/GPA, especially in the early stages of disease course.

Key Issues

- Granulomatosis with polyangiitis (previously known as Wegener's granulomatosis [WG/GPA]) is a granulomatous autoimmune inflammatory disorder of unknown etiology, and has a strong association with cytoplasmic-antineutrophil cytoplasm antibodies and anti-proteinase 3.
- Its etiology is still unknown; however, there is growing awareness of the disease, with an increasing trend in prevalence and incidence observed in the general population.
- Both cellular and humoral immune responses appear to be involved in the pathogenesis of WG/GPA.
- WG/GPA can affect any organ system, but has a predilection for the upper and lower respiratory system and kidneys.
- Ocular manifestations can be the initial presentation of WG/GPA, occurring in 8–15% of patients.
- Proptosis and necrotizing scleritis are the most common ocular complications of WG/GPA, and adequate management of the systemic disease is necessary for their resolution.
- The emergence of new treatment regimens using biologics such as rituximab is recently gaining popularity as the first line of treatment in the management of WG/GPA.

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OriginalArticle

Histopathologicalfeaturespredictiveofaclinicaldiagnosis ofophthalmicgranulomatosiswithpolyangiitis(GPA)

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Abstract: Background:ThelimitedformofGranulomatosiswithPolyangiitis(GPA),formerlyknownasWegener'sGranulomatosis(WG)primarilyinvolvestheheadandneckregion,includingtheorbit,butisoftenadiagnosticchallenge,particularlyasitcommonlylackspositiveanti-neutrophilcytoplasmantibody(ANCA)titresorclassicalfeaturesondiagnosticorbitalbiopsies.ThepurposeofthisstudywastorelatebiopsyfindingswithclinicaloutcomeandtodeterminewhichhistopathologicalfeaturesarepredictiveofaclinicaldiagnosisofGPA.Methods:Retrospectivecaseseriesof234patientsidentifiedfromthedatabaseoftheUCLInstituteofOphthalmologyDepartmentofEyePathologyashavinghadorbitalbiopsiesoforbitalinflammatorydisordersperformedbetween1988and2009.Clinicalrecordswereobtainedforthepatientsandanalysedtoseewhetherpatientshad GPAornot,accordingtoastandardsetofdiagnosticcriteria(excludinganyhistopathologicalfindings).BiopsyfeatureswerethencorrelatedwiththeclinicaldiagnosisinunivariateandmultivariateanalysestodeterminefactorspredictiveofGPA.Results:Ofthe234patients,36werediagnosedwithGPAand198withotherorbitalpathologies.Themajorityofbiopsieswerefromorbitalmasses(47%).Histologyshowedarangeofacuteandchronicinflammatorypicturesinallbiopsies,butthepresenceofneutrophils(P<0.001),vasculitis(P<0.001),necrosis(P<0.001),eosinophils(P<0.02)andmacrophages(P=0.05)weresignificantlyassociatedwithalaterclinicaldiagnosisofGPA.Inamultivariateanalysis,onlytissueneutrophils(OR=3.6,P=0.01)andvasculitis(OR=2.6,P=0.02)wereindependentlyassociatedwithGPA,incontrasttopreviousreportsassociatingeosinophilsandnecrosiswiththediagnosis.Conclusions:Neutrophil,eosinophilandmacrophageinfiltrationoforbitaltissues,togetherwithvasculitisandnecrosis,areallassociatedwithaclinicaldiagnosisofGPA,butonlyneutrophilinfiltrationandvasculitisareindependentlyassociatedwiththisdiagnosis.Thesefeaturesmayassistintheestablishingthediagnosisoflimited GPAamongpatientswithearlyorbitaldisease,particularlyintheabsenceofpositiveserumANCAtitres.

Keywords: Granulomatosiswithpolyangiitis,histopathology,eosinophils,nucleardust

Introduction

Granulomatosis withPolyangiitis(GPA),formerlyknownasWegener'sgranulomatosis(WG),isasystemicsmall-vesselvasculitisthatischar-acterised bynecrotisinggranulomatousinflammationaffectingtherenal,pulmonary,upperairwaysandocularsystems,andisassociatedwithcirculating anti-neutrophilcytoplasmantibodies(ANCA)[1].TheinitialsymptomsofGPAareobservedintheheadandneckregionin some95%ofpatients,butmakingthediagnosisatthisstageisoftenextremely challenging.GPAremainslimitedtoENTandocularinvolvementinsomepatients,butcaninvolveotherorgans, such asthelungs andkidneys [2],withsevereconsequencesforthepatient.Important issuesinthe managementof GPA includebothpredictionofdiseaseprogressionandtheside-effectsofthedrugsrequiredtotreatactivedisease.EarlydiagnosisoflimitedGPAisuseful,asitenablesearlierintroductionoftheappro-

priateimmunosuppressivetherapy,presumablyreducingtheriskoflocalprogressionofthedisease andofinvolvementofotherorgans,includingthekidney,afterwhichtheprognosisdramaticallyworsens[3].

Ocularinvolvementoccursin50-60%ofpatientsandisthepresentingfeaturein8-16%,withsevereocularmorbidityoccurringasacomplicationofbothlocalisedandsystemicGPA[4].Orbitalinvolvementoccursinapproximatelyhalfofallpatientswith GPA,andmayleadtoorbitalbonedestructionandlossofvisionfromcontractionandfibrosisaroundtheopticnerve,despitetreatmentandresolutionofinflammation[5].OcularfeaturesofGPAincludescleritis,whichcanbenecrotisingandbilateral,andwhichcan pose amajorthreattotheintegrityoftheglobe.Nevertheless,thediagnosisofocularGPAisparticularlydifficultasitsclinicalmanifestationsoftenoverlapwithother inflammatory conditions such assarcoidosisandidiopathicinflammatoryorbitaldisorders.Inaddition,inthelimitedformofGPA,antineutrophilcytoplasmantibody(ANCA)titresareposi tiveinonly50-65%ofpatients[6,7].

Althoughthehistopathologicalfindingsareoftendiagnosticforin renaldisease[8,9],thisisnotthecaseforor bitalandupperairwaysdisease,withclassicfindingsbeingpresentinlessthanathirdofpatients,andfewerthanthisinrecentonsetdisease[10,11].Indeed,thehistologicalfeaturesofGPAcanmimicotherformsofidiopathicorbitalinflammationsuchassarcoidosis,idiopathicinflammatoryorbitaldisordersandlymphoidhyperplasia.

ThehistologicalfeaturesoforbitalGPAarediverseandcanincludeanyofthefollowing:granulomatousfoci,collagendeposition,necrosis,nucleardust,plasmacellsandaninfiltratingeosinophilicresponse[12].Thelatterhasbeensuggestedtopredictdiseaseprogression[13],analogous tothesuggestionthat, inrenalbiopsiesinGPA,anincreaseCD8^{+}cellsmightalsopredictandincreaseindiseaseactivity[14].Thesedescriptionsaredrawnfromrelatively small numbersofpatients,and,asyet,therehasnotbeenastudywhichhassystematicallyanalysedalargenumberoforbitalbiopsies.Wethereforeperformedsucharetrospectiveinvestigation for aconsecutivecaseseriesinwhichweexaminedhistopathologyreportsofallpatientswhohadundergoneorbitalbiopsiesfororbitalinflammatorydisorders overaperiodof21years,andrelatedthistotheirclinicalout-comes.
Toourknowledge,thisisthefirststudyofitskind.

Methods

ThestudywasapprovedbytheMoorfields&WhittingtonResearchEthicCommitteeandwasaretrospectivestudyofallpatientswhohadundergoneorbitalorad nexalbiopsyfororbitalinflammatorydiseaseatMoorfieldsEyeHospitalover21years.Allpatientswhohadundergoneanorbitaloradnexalbiopsybetween1988and2009wereidentifiedfromtheUCLInstituteofOphthalmologyDepartmentofEyePathologyDatabase.Thebiopsyreportswerereviewed,andthecelltypesandtissueresponsesdocumentedqualitativelyaspresentorabsent.Celltypesnotedincludedneutrophils,eosinophils,plasmacells,multinucleatedgiantcells,macrophagesandmastcells;tissueresponses assessedincludednecrosis,sclerosis,lymphoidfollicles,nucleardebrisandgranulomas.

Acomprehensivereviewoftheclinicalnoteswasnextperformedtoobtaininformationonpatientdemographicsandclinicaldataincludingthefinalclinicaldiagnosis,orbitalsignsandsymptoms,ocularsignsandsymptoms,otherorganinvolvement,ANCAstatus,treatmentanddurationoffollowup.Patientswereclassifiedashavingclinicalfeaturesconsistentwithadiagnosisof GPAiftheymetanytwoofthefollowingclinicalcriteria:(1)Characteristicocularfeaturesof GPA;(2)CharacteristicENT,pulmonary,renalorcardiacinvolvement;(3)Positiveimmunofluorescencefor ANCA;(4)PositiveELISAforanti-PR3antibodies.Crucially,histologicalfindingsanddiagnosiswereexcludedfromthelistofdiagnosticcriteriatoavoidconfoundingthestudyandpatientswereincludedin the“non-GPA”group if they didnot meet theabovecriteria.SystemicandlimitedGPAweredefinedbythepresenceorabsenceofrenalorlowerairwayinvolvement,respectively.Patientswerealsoclassifiedas‘newly-diagnosed’or‘known’GPAdependingonwhethertheirclini-cal diagnosis had been made prior to the biop-syprocedure.

Table1. Patientcharacteristics

	GPA (n=36)	NonGPA(n=198)	P
Age	50.7 ± 3.2years	50.4 ± 1.2years	0.92
Gender (M:F)	21:15(57%:43%)	127:71 (64%:36%)	0.57
Biopsystructures			
- Orbital mass	20	91	0.29[a]
- Lacrimalgland	8	79	
- Nasolacrimal/sinonasal	12	31	
- Extraocularmuscle	2	28	
- Other	3	11	

[a]Pvaluerepresents χ^2testoforbitalmassvs.otherbiopsysite foreachgroup

Table2. Comparisonofcellularprofiles

Cell type	GPA(%)	NotGPA(%)(n=36)	Unadjusted		Adjuste	d
			OR	P	OR	P
PMN	19 (53%)	30 9(15%)	5.9(2.7 – 12.6)	<0.0001*	3.9(1.4 –11.2)	0.01*
Eosinophil	19 (53%)	62(31%)	2.5(1.2 – 5.0)	0.02*	0.7(0.24– 1.8)	0.42
Vasculitis	19 (53%)	11 (6%)	19.0(7.8– 46.4)	<0.0001*	4.8(1.6 –14.7)	0.006*
Necrosis	22(39%)	21(11%)	5.4(2.4 – 12.0)	<0.0001*	2.4(0.8 – 6.6)	0.10
Lymphocyte	19 (53%)	113(57%)	0.8(0.4 – 1.7)	0.86		
Fibrosis	22(61%)	111 (56%)	1.2 (0.6 – 2.6)	0.72		
Macrophage	16 (44%)	55(28%)	2.1 (1.0 – 4.3)	0.05*	2.0(0.9 – 4.5)	0.11
Plasmacells	18 (50%)	85(43%)	1.3 (0.7 – 2.7)	0.47		
Giantcells	2 (6%)	23(12%)	0.4(0.1– 2.0)	0.39		
Follicles	4(11%)	34(17%)	0.6(0.2 – 1.8)	0.47		
Nucleardebris	2 (6%)	6 (3%)	1.9 (0.4 – 9.7)	0.36		
Granuloma	12(33%)	45(23%)	1.7 (0.8 – 3.7)	0.21		

+ve =cellsreported presentinbiopsy; -ve =cells not reported inbiopsy;OR= oddsratio;CI= confidence interval; *=statistically significantdifference(P<0.05);%= percentage ofpatientswith positive occurrenceofcelltypeortissue responsereportedinbiopsywithineach group.Adjusted odds ratiosare adjusted for allstatisticallysignificant variables (P<0.05) fromtheunivariateanalyses.

Theunpairedstudent'sT-testandFisher'sexacttestwereperformedtoexamine differences between the two groups. Univariate analysis was performed, including the calculation of 95% confidence intervals. Multivariate analysis was performed to look for any independent association of a cellular profile or tissue response with the clinical diagnosis of GPA. For the multivariate logistic regression analyses, all statistically significant variables (P<0.05) from the univariate analyses were included. SPSS 17 and GraphPad Prism 5.01 were used to perform the statistical analysis.

Results

Two hundred and thirty-four patients were identified from the UCL Institute of Ophthalmology pathology database as having undergone orbit-

albiopsyforanorbitalinflammatoryconditionbetween1988and2009,andforwhomfullclinicaldetailswereavailable.Ofthese,36patientsfulfilledourcriteriaforthediagnosisofGPA.DiseasesinthenonGPAgroupincludedchronicidiopathicinflammationoftheorbit(CIIO)in74/198patients(37%),lymphoidhyperplasiain16(8%),sarcoidosisin13(7%),myositisin3(2%),dacroadenitisin33(17%),andthyroideyediseasein12(6%).Patientcharacteristicsareincludedin**Table1**,andindicate no significantdifferences betweentheGPAandnonGPAgroups.

ClinicalcharacteristicsoforbitalGPA

Ofthe36patientsintheGPAgroup,twopatients(6%)hadsystemicGPA(thatis,withrenalorlowerairways involvement)and34

(94%)hadlimiteddisease.Seventeen(47%)hadbeendiagnosedwithGPAbeforetheirocularsymptomscommencedandhadreceivedtreatmentintheformofcorticosteroids,withorwithoutsecondlineimmunosuppression;theremaining19(53%)hadadiagnosisofGPAbasedontheiroculardisease.Themostcommonocularpresentationsin theGPAgroupwereproptosis(55%),lidswelling(44%)andscleritis(32%).Otherocularpresentationsincludeddiplopia,epiphora(nasolacrimalblock),ocularpainanddecreasedvision.Interestingly,andinagreementwithrecentstudies,nopatientswithlimitedGPAwereobservedtoprogresstothesystemicformofGPAduringthedurationofthisstudy[15,16].Thedurationoffollow-upinthisstudywasamedian36months(range24to190months;708patient-yearsintotal).

Biopsyfeaturespredictiveofclinicalfeaturesconsistent withadiagnosisof GPA

Intheunivariateanalysis,neutrophils,eosinophils,vasculitis,macrophagesandnecrosisrepresentsignificantly moreoftenintheGPAgroupthaninthenon-GPAgroup(**Table2**).Amultivariateanalysis,controllingforconfoundingfactors,showedthatneutrophilsandvasculitis areindependently associated withtheclinicaldiagnosisofGPA-withoddratiosof 3.9and4.8respectively.

TwelvepatientswereANCA-positive,11wereANCA-negativeandtheremaining13patientsdidnothaveanyANCAlevelsonrecord–beingpatientswithanestablisheddiagnosisofGPApriortotheirophthalmicpresentation.Therewasatrendforeosinophils(P=0.08)andlymphocytes(P=0.09)tobeseenmorefrequentlyintheANCA-positivegroupthanintheANCA-negativegroup.

Justoverahalf(19/36)ofthepatientswerenewlydiagnosedwithGPAand17hadaknowndiagnosisofGPApriortoophthalmicpresentation,including2patientswithsystemicGPA.AllpatientswithestablishedGPAhadpreviouslyreceived,orwerestillon,immunosuppressivetreatment.Presumablyduetoalackofpriortreatment,cellular infiltration was greater inpatientswithoutapriordiagnosisofGPAandthechangesincludingthepresenceofnucleardebrisandgiantcells,bothofwhichwereseenonlyinpatientswithoutpriortherapy.

Discussion

ThediagnosisofophthalmicGPAisdifficult,asitsclinicalmanifestationsoftenoverlapwithother inflammatorydisorders. Furthermore,ANCAtitresarepositiveinonly50-65%ofsuchpatients,andclassichistologicalfeaturesarealsofrequentlyabsentfrombiopsymaterial;thisbeingincontrasttodiseaseaffectingotherorgans,suchasthekidney[5,7,11,13].Nevertheless,thisinvestigationindicatesthattherearesignificantdifferencesinthecellularandtissueprofileinbiopsiestakenfrompatientswhowilllaterbeassignedadiagnosisofGPA,ascomparedtootherdiseases,thepresenceof infiltratingneutrophils and vasculi-tis beingindependentlyassociated witha clinicaldiagnosisofGPA.BiopsiesfrompatientswithGPAalsoshowmanymoreinflammatorycellsascomparedtoothertypesoflymphocyticorbitalinflammatorydisorders,whichreflectsthetendencytoamorefulminantdiseasewithGPA.

Previousstudieshavesuggestedthateosinophilsplayapartinthepathogenesisof GPAand,inonestudy,thepresenceofeosinophilsinbiopsiesofpatientswithlimitedGPAwassaidto bepredictorofdiseaseprogression [7].Inourstudy,aunivariateanalysisdidindicatesignificantlymoreeosinophilsinpatientswithGPA,butthiswaslostwhenotherconfoundingfactors were controlledfor,suggesting thatthenumberoftissueeosinophilsmightbeassoci-atedwithdiseaseseverity,ratherthanbeingassociatedwithGPAinparticular.

Nucleardebrisisanentitythathasrecentlybecomeasubjectofinterestin GPAandisthoughttooriginatefromtheruptureofnucleiofneutrophilsinthetissueofpatientswithGPA,possiblyasaresultofANCAactivity[9,12].Inourstudy,nucleardebriswasseenmorefrequentlyintheGPAgroup,butthismightbeunderrepresentedaspathologistsmaynothavespecificallyreportedthisphenomenoninthepast;furtherquantitative evaluation ofthisentityinorbitalbiopsieswouldbeinterestingandbeneficial.Inaddition,the efficacyofritux-imabincertainpatientswithGPAimplicatesaroleforBcellsintheaetiologyandperpetua-tionofdisease, and furthercharacterisation ofthesecellsatdifferentstagesofdiseasewouldbeofinterest [5,17,18].

Interestingly,despitethelimitationthatallofthecellularprofilesinthisstudywerebasedonhistopathologicalreports,wewerestillabletodetectsignificantdifferencesincellularactivitybetweentheGPAandnon-GPAgroup.Developingagradingsystemforthecellularandtissueprofilesshouldprovideaclearerpictureofcellularactivity in these orbital biopsies, and thereby enableamoreaccuratecomparisontobemadebetweenorbitalbiopsies.

Ethicalapproval

The study is approvedby the Moorfields &WhittingtonResearch EthicCommittee (RECref. no.09/H0721/75,LIGS 1023).

Declarationofcompeting/conflictsof interest

Wedeclarethatallauthorshavenocompeting/conflictingofinterestsmightbeperceivedtoinfluencetheresultsand/ordiscussionreportedinthisarticle.

Declarationoffunding

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Diagnostic value of IL-17, IL-23 and BAFF-R in localised granulomatosis with polyangiitis (submitting)

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We verify that the manuscript submitted, including related data, figures, and tables, has not been previously reported or published; that the manuscript is not under consideration elsewhere; and that it will not be submitted elsewhere while under review by the JCI.

Abstract

Histology investigation from orbital biopsies plays an important role in the diagnosis of orbital granulomatosis with polyangiitis (GPA). However classical histological features of GPA in orbital tissues may not always be present and the histology picture regularly appear similar to other orbital inflammatory diseases (OID). Despite this, disease progression in orbital GPA usually runs a more severe course compared to OID. Subjective histopathology reports have shown that biopsies of orbital GPA appear to have higher cellular activity when compared to OID. In this study, we performed Haematoxylin and eosin (H&E) and immunohistochemistry stain to objectively count and compare the cellular activities and tissue changes between orbital biopsies of GPA and other OIDs. We also observed for any distinctive feature in the histology for orbital GPA. Our findings objectively confirmed that the inflammatory activity was more in orbital GPA compared to other OID. However, no distinctive feature for the diagnosis of orbital GPA was identified. Nevertheless, cytokines IL-17 and IL-23 and B-lymphocytes with B-cell activating factor receptors (BAFF-R) were seen significantly more in orbital GPA, suggesting that they may have a large influence in the pathogenesis of GPA and can potentially be of value in the diagnosis of this disease.

Introduction

Granulomatosis with polyangiitis (GPA), previously known as Wegener's granulomatosis, is an idiopathic granulomatous inflammatory disease that forms part of the spectrum of anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis(1). Pathologically, the disease is characterised by granulomatous inflammation, vasculitis involving small and medium vessels and patchy necrosis. GPA can generally be divided into two forms: systemic GPA or localised GPA. Systemic GPA refers to major organ involvement, in particular renal disease, and can be life-threatening. In contrast, localised GPA is more confined, involving one or two organs, such as the eye and/or the upper respiratory system and is generally not life-threatening. In up to 95% of cases, patients present with head and neck disease(2). Ocular involvement occurs in 50-60% of patients (3) and is the presenting feature in 8-16%; severe ocular morbidity may be a complication of both localised and general GPA(3).Orbital involvement is present in about a half of patients with GPA, which may be secondary to spread from the sinuses, occur as part of systemic GPA or just localised to the periocular area; simultaneous orbital and intraocular disease occurs only rarely.

The diagnosis of GPA is often challenging, especially if localised (such as orbital GPA), as the clinical presentation maybe non-specific and often indistinguishable from other orbital inflammatory diseases (OIDs) such as idiopathic inflammatory orbital disease (IIOD) and orbital sarcoidosis. Serologic testing for serum anti-neutrophil cytoplasm antibody (ANCA), an antibody intimately associated with GPA, is usually advocated to aid diagnosis but with localised disease ANCA is often negative at presentation and is detected in fewer than two-thirds of patients with time(4)(5), as compared to 90% in those with generalised disease(6).Although imaging is useful in GPA to assess the extent of disease, its diagnostic value is limited.

Histological examination plays an important role in the diagnosis of GPA and should be used where possible. The absence of major vessels in orbital biopsies can, however, make the diagnostic vasculitic changes hard to find in orbital biopsies from patients with localised GPA; in nasal and orbital biopsies, for example, typical histological findings are seen in less than a third of patients(7). In most patients with localised GPA, the difficulty in interpretation of the tissue biopsy and the low rate of ANCA positivity not uncommonly leads to a significant delay to diagnosis and possibly and consequential risk of widespread disease.

Orbital involvement in GPA can lead to more severe disease than other causes of orbital inflammation.A recent report observed more neutrophil infiltration in biopsies from patients with GPA but did not find any distinctive features diagnostic for orbital GPA(8).Sarcoidosis is also characterized by granulomatous inflammation but the disease course of orbital GPA and sarcoidosis can bedissimilar, the latter tending to cause chronic lacrimal gland inflammation with a lower incidence of more widespread or posterior disease.

These clinical observations suggest that different inflammatory pathways occur in GPA as compared with other inflammatory orbitopathies, with T-cell CD134 (cluster of differentitation 134) and T-helper 17 (Th17) markers, as well as B-cell markers, implicated in the pathogenesis of GPA(9)(10)(11)(12). CD134, also known as OX40, is a member of the tumour necrosis factor receptor (TNFR)family of receptors and plays a critical role in maintaining an immune response by preventing activated T-cell death. Th17 cells, producing interleukin (IL) 17, play an important role in inducing and mediating a pro-inflammatory response by enhancing T-cell priming and stimulating the production of other pro-inflammatory molecules (including IL-1, IL-6, and tumor necrosis factor (TNF)). IL-23 is known to stimulates naive CD4-positive T-cells to differentiate into Th17 cells and has been associated with other autoimmune diseases, such as Crohn's disease (13) as well as ANCA-associated vasculitis (AAV) (14).B-cell activating factor (BAFF), also known as B lymphocyte stimulator (BLyS) or "tumour necrosis factor ligand superfamily member 13C", belongs to the TNF ligand family and is a ligand for the BAFF receptor (BAFF-R). BAFF is a potent B-cell activator and plays a role in the proliferation and differentiation of B-cells during an immune response. All such studies report, however, surrogate markers in the serum and the tissue levels of pro-inflammatory chemokines and cytokines remains unknown.

In this study we investigate thedifferences in cellular infiltrates in orbital tissuesfrom patients with orbital GPA and other OID, by objectively comparing and quantifying the different T-cell types and tissue changes. Weused immunohistochemistry (IHC) to investigate expression of tissue cytokines, to determine whether IHC could have a role in early diagnosisof orbital inflammatory diseases. The main findings of the study arethat the tissue expression of IL-17, IL-23 and BAFF-Raresignificantly greater among patients presenting with orbital GPA than in patients diagnosed with sarcoidosis and IIOD, suggesting a possible early diagnostic role for such 'sentinel' biomarkers.

Method and materials

Ethical approval

The study was approved by the Moorfields& Whittington Research Ethics Committee (REC ref. no. 09/H0721/75, LIGS 1023).

Patient selection

All patients who, between 1988 and 2009, had undergone biopsy of periocular tissues for orbital inflammatory disease were identified from the Institute of Ophthalmology Eye Pathology Database and their case notes reviewed. The biopsy was only included in this study if the diagnosis and management of their orbital disease was based on their having features from two or more of the following groups: clinical history, clinical manifestations, biochemical investigations or radiological features. Biopsies from patients in whom the diagnosis and management of their orbital disease wasbased entirely on the histological appearance were excluded from this study.

Tissue selection

Paraffin blocks of tissue biopsies were retrieved from archive at the Department of Eye Pathology, UCL Institute of Ophthalmology.Paraffin blocks were anonymised by using the pathology numbers that matched an allocated patients' study number. Haematoxylin and Eosin (H&E) staining was performed on all blocks for general cellular analysis and then, for specific immunohistochemical (IHC) staining, patients were further divided on the basis of their clinical diagnosis. Paraffin blocks of 25 patients from the orbital GPA group and 25 patents from the IIOD group were randomly selected for IHC analysis. Due to insufficient tissue in one case, only 13 blocks from the 14 patients with a clinical diagnosis of orbital sarcoidosis were available for IHC.

Antibodies

Antibodies used are listed in Table 4

Slide preparation and H&E staining

Five μm thick tissue sections were cut using a sledge microtome and mounted on Superfrost-plus object glass slides. The slides were left on a hot plate at 40°C for 60 minutes and then incubated overnight at 37°C before use. Haematoxylin and eosin (H&E) staining was performed on all slides using a standardlaboratory protocol.

Immunohistochemistry staining of fixed paraffin tissues

Tissue sections were dewaxed in two changes of xylene (each of 5 to 10 minutes) with agitation at regular intervals, then twice with 100% ethanol (10 to 20 seconds), and then hydratedsequentially in 90%, 70%, and 50% ethanol -- each for 10 to 20 seconds -- followed by two washes in water. Sections were incubated in the appropriate antigen retrieval solution: Tris/EDTA pH 9.0 (H-3301, Vector Labs, UK) for CD3, CD4, CD20 and CD68; sodium citrate pH 6.0 (H-3300, Vector Labs, UK) for CD134, IL-17 and IL-23); and heat-induced epitope retrieval was performed using pressure cooker at 95°C. Slides were then cooled under running water and left immersed in Tris-buffer solution (9L distilled water + 1L DAKO Wash Buffer 10X (Code S3006; DAKO UK) for 5 minutes. CD3 (1:400), CD4 (1:50), CD8 (1:50), CD20 (1:200) and CD68 (1:100) stainings were performedby the DAKO Autostainer Plus.

For CD134 (1:200), IL-17 (1:800), BAFF-R (1:50) and IL-23 (1:400), IHC preparation was performed manually: After de-waxing and antigen retrieval *(v.s.)*, the slides were placed in a moist chamber, the tissues kept hydrated with Tris-buffer solution, and the tissue encircled using a hydrophobic barrier pen (ImmEdge Pen H-4000). Primary antibodies, diluted in blocking solution, were added and left to incubate overnight at 4°C. Washing with Tris-buffer was performed after each incubation step. The LSAB+, Dako REAL™ Detection Systems kit (K5001 HRP/DAB+, Rabbit/Mouse or K5005 AP/RED, Rabbit/Mouse; DAKO, UK) was used for visualization. Finally, the slides were counterstained with haematoxylin, mounted with DPX mounting medium and cover slips, and then allowed to dry overnight before imaging.

Imaging and image analysis

Haematoxylin &Eosin

Images of whole slides were acquired using the Hamamatsu Nanozoomer Digital Pathology Scanner and the counts for cell-types and tissue changes were obtained using the ImageScope image analyser and ADCIS ® Stereology Toolkit. Cell counts were performed for neutrophils, eosinophils, lymphocytes, macrophages, plasma cells, giant cells and mast cells. Tissue changes characterized were nuclear debris, granulomas, vasculitis, necrosis and fibrosis.

The ADCIS ® Stereology Toolkit frame count programme was used for cell counting: initially the border of the whole tissue section was defined manually and then the region of interest was identified within the image of the tissue section. The software was programmed to generate 20 random counting frames within the region of interest, each counting frame measuring 1mm x 1mm and consisting of two inclusion lines (right and top borders) and two exclusion lines (left and bottom borders). A total area of $20mm^2$ was therefore analysed for every slide, with cells inside the frame and not touching the exclusion lines being included in the count. In all 20 frames, each cell type was identified manually and marked using different specific colours. The computer then assessed the total number of marked cells for each cell type, to generate the total counts.

For tissue changes, the ADCIS ® Stereology Toolkit point count programme was used. The region of interest was identified from the slide image as previously described and the software was then programmed to generate 20 random points (marked with X) within the region of interest. Different colourcodings were assigned to different tissue changes and eachof the 20 randomly-chosen point was marked accordingly if that particular tissue change was present. The computer then summates the number of 'hits' for each tissue change.

Immunohistochemistry

The ADCIS ® Stereology Toolkit frame count programme was used as before but, for IHC, 50 counting frames (instead of 20) were randomly generated by the software, giving a total analysis of $50mm^2$ for every slide. The total number of marked cells was censused using the same edge-convention for counting.

Data analysis

Cell counts were entered into an Excel spreadsheet. For H&E analysis the patients were classed as clinically-diagnosed orbital GPA or non-GPA (other OIDs), and cell and tissue counts were compared. Further sub-analysis was also performed within the orbital GPA group. For IHC, quantitative comparisons of the individual stained cells were done between the GPA, IIOD and sarcoidosis groups.

Statistical analysis

The non-parametric count comparisons for general cellular and tissue analysis (H&E stains)between orbital GPA and non-GPA were performed using Mann-Whitney tests with SPSS17. Multivariate analysis was carried out with SPSS17 to look for any independent association between the cellular profile or tissue response and the diagnosis of GPA.

Kruskal-Wallis test was performed using GraphPad Prism 5 for comparisons of the non-parametric IHC cell counts between orbital GPA, IIOD and orbital sarcoidosis, and a secondary analysis used Dunn's Multiple Comparison Test to compare groups.

Results

A total of 234 patients with a clinical diagnosis of orbital inflammation were identified from the Institute of Ophthalmology(UCL)Eye Pathology database, and fulfilled the inclusion criteria (see methods); 239 tissue blocks of orbital biopsieswereretrieved. With review of the pathology, the final diagnoses were GPA 39 (16%), IIOD 65 (27%), dacryoadenitis 35 (14%), lymphoid hyperplasia 23 (10%), sarcoidosis 14 (6%), thyroid eye disease 12 (5%), orbital myositis 4 (2%); the diagnoses in the remaining 47 specimens included ruptured dermoid cyst, dacryocystitis, mucocoele, orbital xanthogranuloma, Churg-Strauss syndrome and post-surgical granulomas. Tissue IHC was performed on 63 orbital biopsies from 63 patients, consisting of25 consecutive specimens where a clinical diagnosis of GPA was secured, 25 specimens from patients with IIOD, and 13 with orbital sarcoidosis(1 had insufficient tissue).

Haematoxylin&Eosin analysis

Orbital biopsies from GPA patients appearedmore cellular thanthose from other OIDs (Figure 1), with the cell-counts for each cell-type and the tissue changes summarized in Table 1. Neutrophils (p=<0.001), eosinophils (p=0.002), macrophages (p=<0.001), nuclear debris (p=0.011), vasculitis (p=<0.001) and necrosis (p=<0.001) were found to be significantly more abundant in the orbital GPA group compared to non-GPA group (Table 1). Multivariate analysis showed that necrosis and vasculitiswereindependently associated with the clinical diagnosis of GPA, with an odds ratio of 2.40 (p<0.001) and 1.33 (p<0.001),respectively. Nevertheless,12/39 (31%) of orbital GPA biopsies did not show any necrosis andin 5 (13%) cases there was neither vasculitis nor necrosis. Granulomas were significantly less frequent in GPA as compared to non-GPA (odds ratio (OR) = 0.12, p=0.02) (Table 1).

Immunohistochemical analysis

Comparisons of IHC staining between three orbital pathologies are summarized in Table 2: CD3 and CD68 expressions showed significant differences between the three diseases, but further analysis showed that the differences were only between GPA and IIOD (Figures 2 and 3).

Three cytokines or receptors; namely IL-17 (p=0.002), IL-23 (p<0.001) and BAFF-R (p=0.008), showed distinct differences between the three pathologies: further analysis (post-hoc) with Dunn's multiple comparisons revealed these three cytokines/receptors wereexpressed significantly more in GPA biopsies as compared to both IIOD and orbital sarcoidosis (Table 3; Figures 4-6**Error! Reference source not found.**). A raised IL-17 count was more likely to be found in orbital GPA than in IIOD or orbital sarcoidosis.Likewise, high IL-23 counts are 9 times more likely in orbital GPAthan IIOD (OR=9.3; CL 1.05-82.8), and not likely to be present in orbital sarcoidosis.Biopsies with a high BAFF-R count were 6 times more likely in orbital GPA, as compared to both IIOD (OR=6.6, CI=0.65-55.7) and orbital sarcoidosis (OR=6, CI=0.65-55.7).

Histology and clinical correlation analysis within GPA group

Of the GPA group, 18/39 tissue samples were from patients newly-diagnosed with GPA and the remaining 21 had known GPA; having been treated for other clinical manifestations before orbital presentation. Patients with known GPA had significantly greater eosinophil counts and a trend towards increased macrophage infiltration (p=0.045 and p=0.06, respectively).

Other comparisons in the GPA group

There was no difference in cell and tissue counts with regards to ANCA status (positive *versus* negative), disease extension (generalized *versus* localized GPA) and treatment regimen (single *versus* multiple therapies).

Discussion

The diagnosis of orbital GPA is challenging and tissue biopsy is anticipated to be important in confirming a suspected clinical diagnosis but this is not always possible. The clinical presentation of orbital GPA can be similar to other forms of orbital inflammation and diagnostic tests, such as serum ANCA and orbital CT, lack sensitivity and specificity.

Histopathology from GPA orbital biopsies show a significantly higher inflammatory activity as compared to other OIDs (8), and this study was designed to quantify and compare the cellular and tissue inflammatory responses in orbital GPA and other OIDs. Based on morphology and IHC markers, the distribution of cell types present were compared across different forms of OID to determine which, if any, might be practical diagnostic biomarkers. Our study showed three biomarkers that are significantly elevated in GPA orbital biopsies – namely,IL-17, IL-23 and BAFF-R that are significantly higher in the orbital biopsies of GPA patients as compared to those with IIODor sarcoidosis.

IL-17 is a pro-inflammatory cytokine produced by Th17 cells, a subset of T-helper cells, that are considered to be distinctly developed from Th1 and Th2 (15). Th17 cells have been reported in many autoimmune and chronic inflammatory diseases, such as rheumatoid arthritis (16), psoriasis (17)(18), Crohn's disease and systemic lupus erythematosus (19). In the eye, Th17 cells have been shown to play a role in ocular inflammations, such as in uveitis and scleritis(20), Behcet's uveitis (21)(22)and the uveitis associated with Vogt-Koyanagi-Harada disease(23).

Th17 cells have also been reported to be instrumental in the pathogenesis of ANCA-associated vasculitis (AAV)(24)(14), particularly GPA. In ANCA-positive GPA patients, Th17 cells were found to be skewed upon stimulation of PR3 (11), and seen to be expanded in both active and quiescent GPA when compared to healthy individuals (10). Our IL-17 finding concurswith these reports and also affirms the possible role of IL-17 in the pathogenesis of GPA. Bronchial tissue granulomas and circulating memory T-cells in sarcoidosis are reported to have an increased expression of IL-17A, this possibly being involved in the induction and maintenance of granulomas (25). Involvement of IL-17A in granuloma induction and maintenance was not mirrored in our orbital GPA biopsies, where higher IL-17A expression was accompanied by few granulomas. This suggests that IL-17 might have a different mechanism of action in the pathogenesis of GPA and sarcoidosis.

The significant increase in IL-23 in GPA,as compared to both IIOD and sarcoidosis, is particularly interesting. IL-23 is produced by macrophages and dendritic cells and its role in inflammation is primarily established as a crucial factor in the development of Th17 and IL-17 cytokine production(26). Nevertheless, IL-23 alone has been shown to induce disease: IL-23 has been shown to inducearthritis and osteoclast formation in animals, with resultant bone destruction, this being independent of IL-17A (27). A similar mechanism might explain the sino-orbital bone destruction seen in GPA, but which does not occur in orbital sarcoidosis or IIOD. IL-23 has also been related to disease severity: in AAV, including GPA,patients with elevated levels of IL-23 had more active disease compared to those with low IL-23 (14).

In autoimmune diseases, IL-23 has been shown to drive pathogenic T-cells that induce autoimmune inflammation by expanding self-reacting IL-17, TNF and IL-6 producing T-cells (28). Indeed, the IL-23/IL-

17 pathway has been shown to be involved in several autoimmune diseases, such as Crohn's disease (13) and Vogt-Koyanagi-Harada disease (23). As compared to healthy individuals, serum IL-17 and IL-23 levels in AAV are significantly higherand remained elevated in a proportion of convalescent patients (14). To our knowledge this is the first report to show the presence of raised IL-17 and IL-23 in inflammatory orbital tissues, and also the first to identify a significant rise in tissue IL-23 in orbital GPA as compared to other forms of orbital inflammatory disease.

Rituximab, a monoclonal anti-CD20 antibody, has been found to be effective in treating refractory AAV (29), including refractory ophthalmic GPA (30), this implicating the role of B-cells in GPA(31). The B-cell activating factor receptor (BAFF-R), expressed on B-lymphocytes, selectively binds cytokine B-cell activating factor (BAFF) (32), a member of the tumournecrosis factor (TNF) ligand family which, like APRIL(a proliferation-inducing ligand), acts as potent B-cell activator and plays an important role in their proliferation and differentiation. BAFF-receptor activation is crucial to the survival and maintenance of mature B-cells (33)(12), and BAFF is known to be elevated in the serum and inflamed musosal surfaces of patients with GPA (34)(35)(12). Serum levels of BAFF are also elevated in chronic active sarcoidosis, as compared to healthy people or patients with inactive sarcoidosis(36). Although CD20 counts in our study were similar between orbital GPA and IIOD, and between orbital GPA and sarcoidosis, orbital GPA tissues were found to exhibit the highest levels of BAFF-R. This suggests that, while B-cells probably have a role in all three diseases, prolonged B-cell survival and the ability of B-cells to remain activemight be a differentiating factor in the manifestations and severity of orbital GPA.

The H&E tissue analysis of this study confirmed our previous report of increased cellular infiltrationin orbital GPA,as compared to other OIDs(8). In addition, this study also showed that vasculitis and necrosis were features independently associated with the diagnosis of orbital GPA. This finding is consistent with the histology of GPA in other organs. Pulmonary necrotizing vasculitis is said to be the single most important feature in the histologic diagnosis of GPA (37) and, likewise, the main histological feature in patients with active renal GPA is segmental glomerular necrosis (38). Together with the higher tissue inflammatory cell infiltrate, the presence of vasculitis and necrosis in GPA orbital tissuesreflect the more severe and destructive nature of orbital GPA, compared to other OIDs. Nevertheless, it is important to acknowledge that vasculitic and necrotic changes might be absent in orbital biopsies although clinically the diagnosis is confirmed to be orbital GPA -- thereby highlighting the importance of biomarkers. The biopsies in orbital GPA often display a more varied and denselycellular appearance than other forms of OID, this histology being mirrored in pulmonary biopsies from GPA -- described as areas with a deeply basophilic, "dirty" appearance due to the presence of neutrophils and nuclear debris (39)(37).

GPA pathology is classically described as a granulomatous inflammation with vasculitic and necrotic features and so, in our study, it was unexpected to find that granulomas were inversely associated with the diagnosis of orbital GPA. A granuloma is typically described as a distinctly organized "ball or nodule-like" collection of macrophages, in response to infection or inflammation. GPA and sarcoidosis are both described as granulomatous inflammations, although this term is probably more consistent with the histology of sarcoidosis, in which discrete, well-formed interstitial non-necrotizing ganulomas are seen (37). In GPA, the term 'granulomatous inflammation' is ill-defined (40) and, even in lungs, the compact, "sarcoid-like," non-necrotizing granulomas are said to be exceptional. Pulmonary granulomas with GPA tend to be suppurative or neutrophil-filled, and have an irregular contour -- unlike the granulomas seen in sarcoid or infection (37). The histology of our orbital GPA biopsies showed an inflammatory picture with abundance of macrophages, neutrophils and nuclear debris, but distinct granuloma formation was rare.

The role of eosinophils inGPA is unclear, although their presence in orbital GPA has been documented(41)(42). We found eosinophilsto be more frequent in orbital GPA as compared to other OIDs, and also to be marginally more in patients with known systemic GPA. This finding might suggest either that the recruitment of eosinophils is higher during recurrent GPA, or that eosinophils in orbital GPA persist despite treatment and are retained in affected peripheral tissues. Thus, eosinophils might be associated not only with disease severity (as indicated in our previous report (8)), but also the chronicity or relapseof orbital GPA.

Conclusion

There exist clear differences in the histology and cellular infiltrates in orbital GPA when compared to other OIDs. The heightened expression of IL-17, IL-23 and BAFF-R seen in the biopsies of orbital GPA could both serve as early diagnostic markers for disease, and also provide new insights into the underlying inflammatory pathways. Early identification of such markers in patients with orbital inflammation could prompt the clinician to consider a diagnosis of GPA and instigate earlier treatment of a potentially sight- and life-threatening disease.

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FIGURES

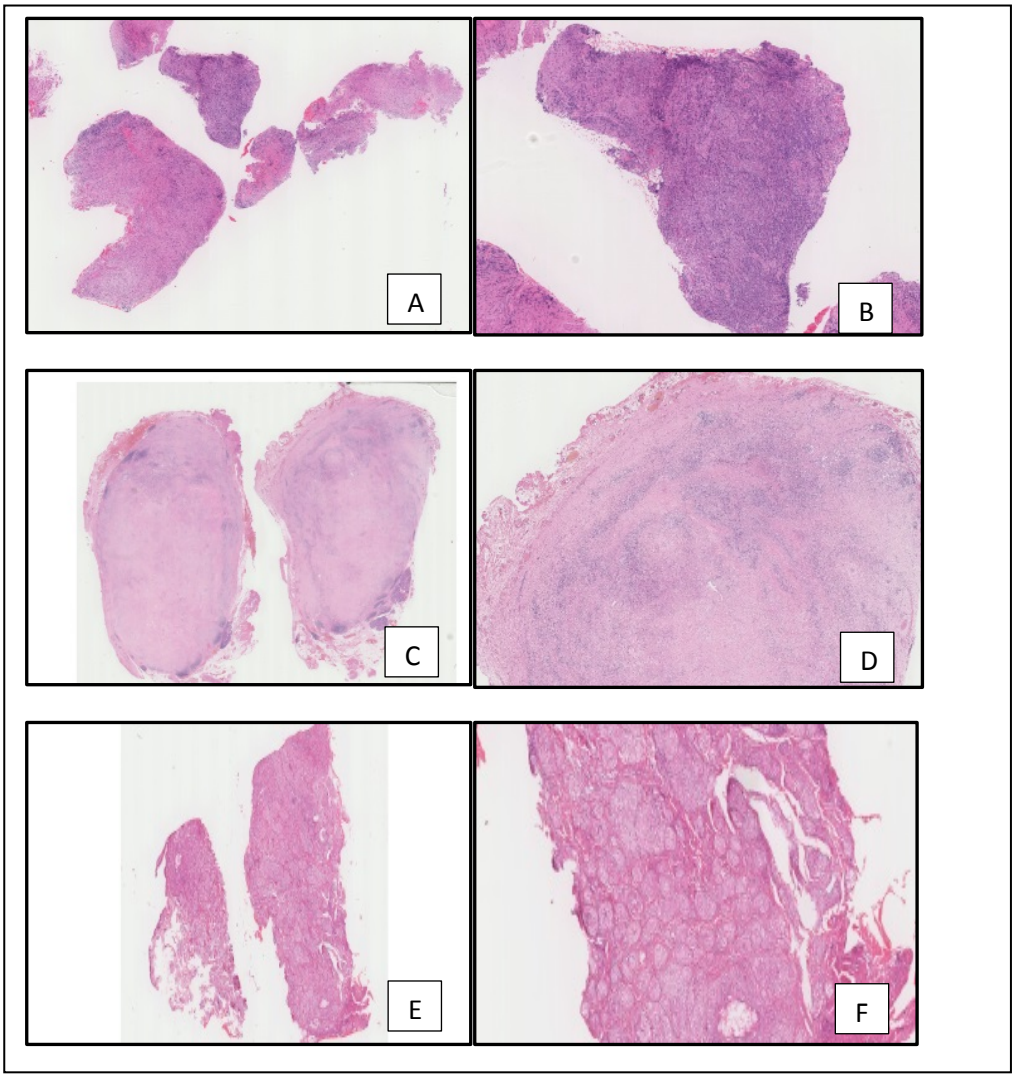

Figure 1 Representative tissues (H&E) showing (A&B)orbital granulomatous polyangiitis with a 'darker' stained tissue and a more densely cellular appearance; (C&D) tissues from patient with idiopathic orbital inflammatory disease; (E&F) orbital sarcoidosis showing distinct granulomas.

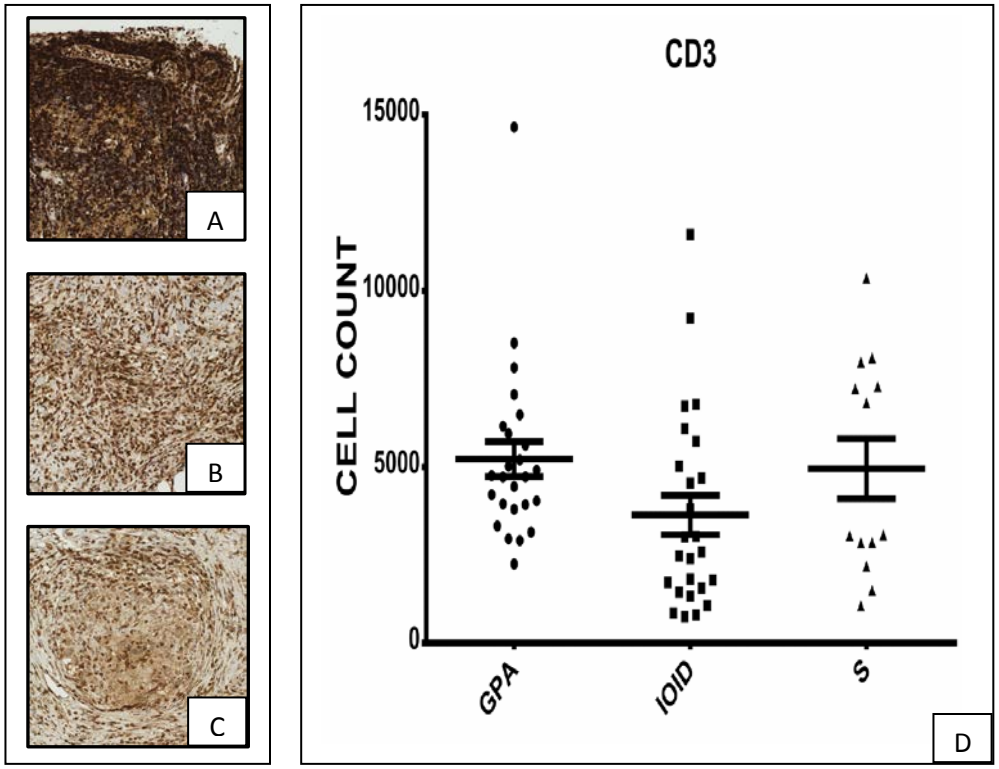

Figure 2CD-3 staining on orbital tissues from patients with (A) orbital granulomatous polyangiitis ("GPA"), (B) idiopathic orbital inflammatory disease ("IOID") and (C) orbital sarcoidosis ("S"). (D) Scatter plot showing the mean count of CD-3 for each of the three orbital diseases.

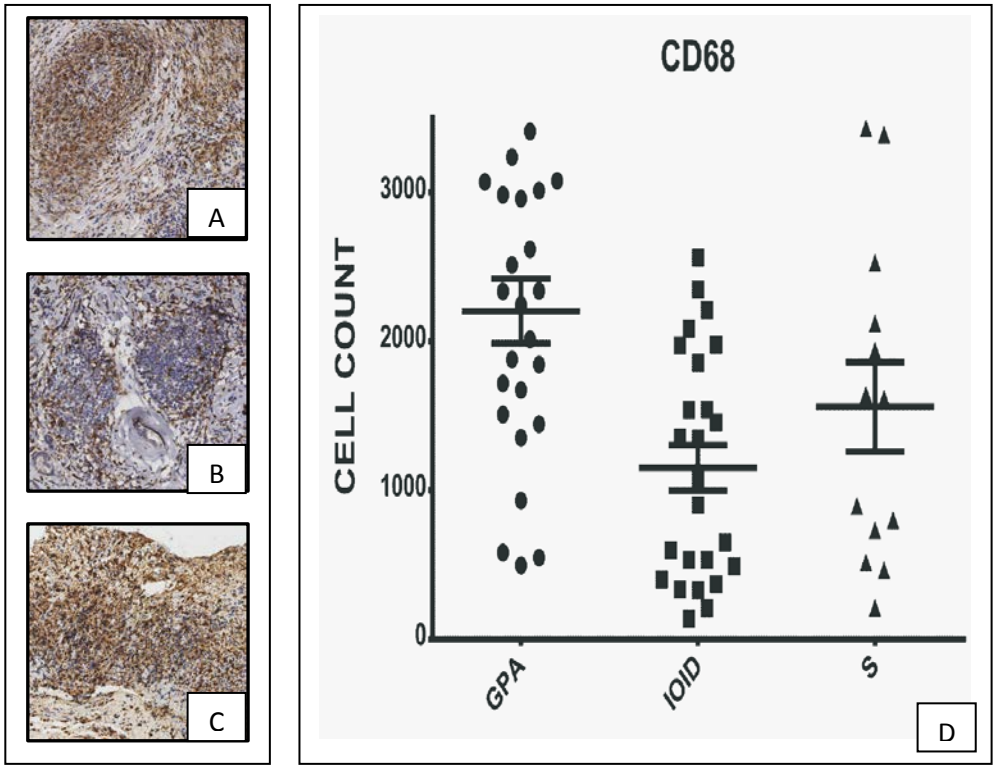

Figure 3CD-68 staining on orbital tissues from patients with (A) orbital granulomatous polyangiitis ("GPA"), (B) idiopathic orbital inflammatory disease ("IOID") and (C) orbital sarcoidosis ("S"). (D) Scatter plot showing the mean count of CD-68 for each of the three orbital diseases.

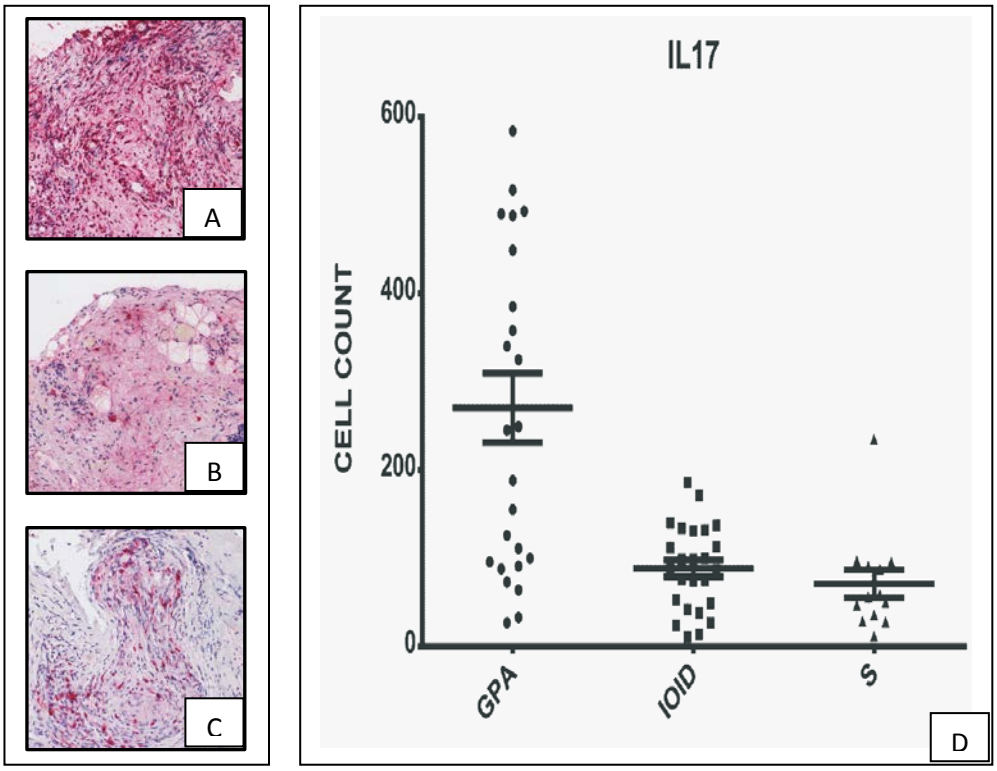

Figure 4IL-17 staining on orbital tissues from patients with (A) orbital granulomatous polyangiitis ("GPA"), (B) idiopathic orbital inflammatory disease ("IOID") and (C) orbital sarcoidosis ("S"). (D) Scatter plot showing the mean count of IL-17 for each of the three orbital diseases.

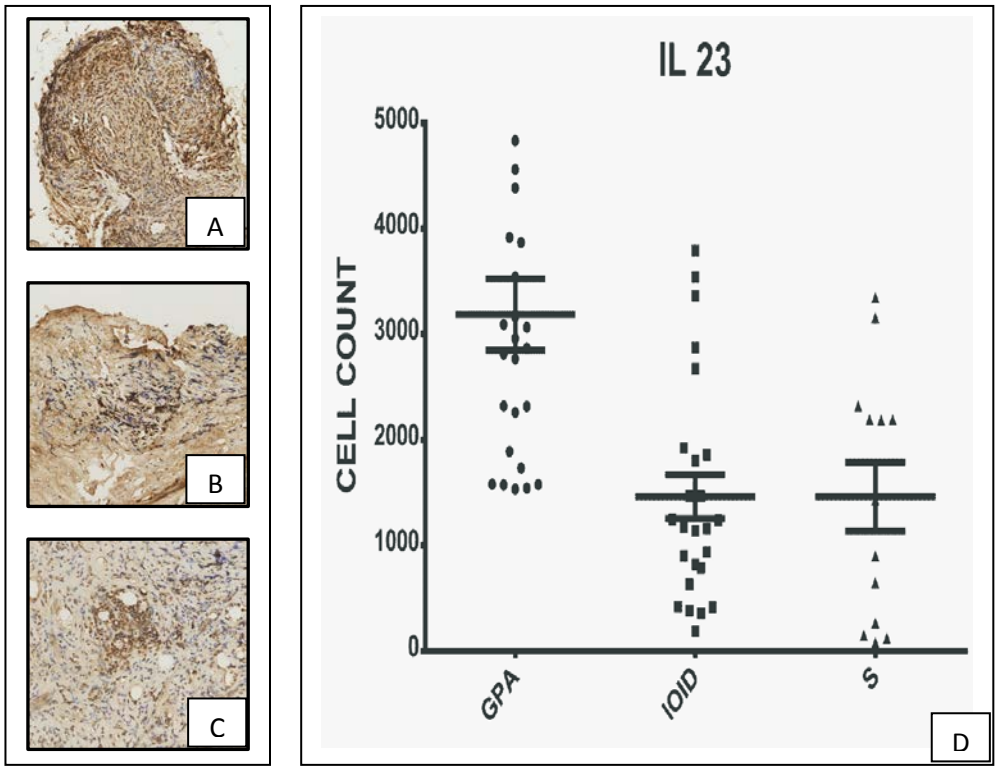

Figure 5IL-23 staining on orbital tissues from patients with (A) orbital granulomatous polyangiitis ("GPA"), (B) idiopathic orbital inflammatory disease ("IOID") and (C) orbital sarcoidosis ("S"). (D) Scatter plot showing the mean count of IL-23 for each of the three orbital diseases.

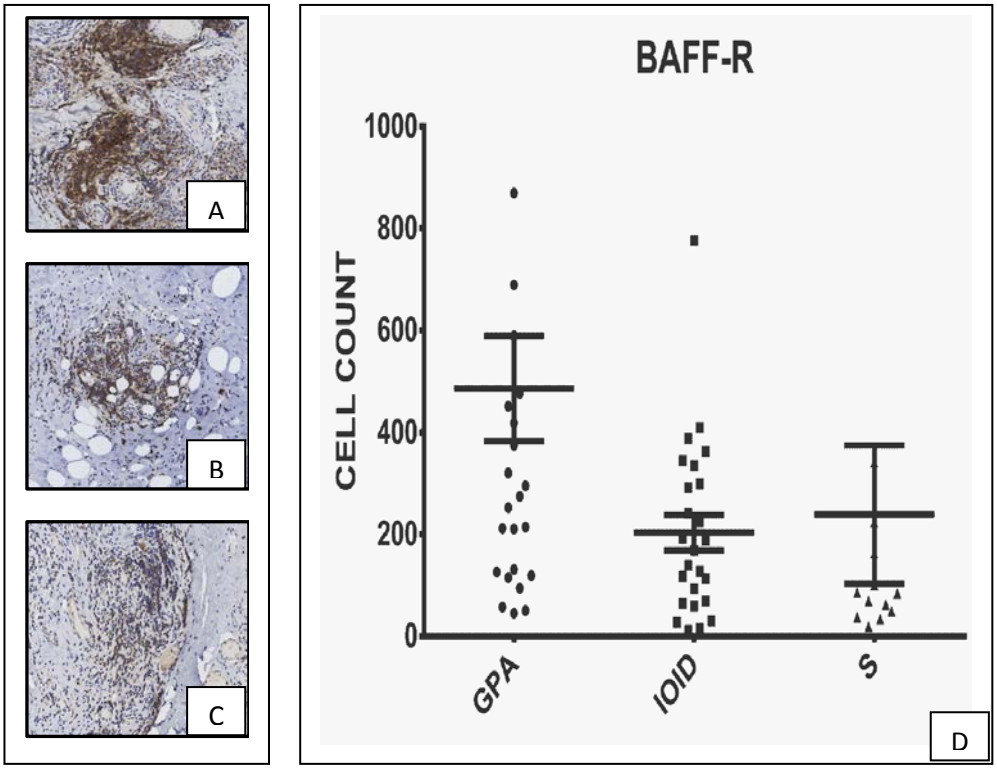

Figure 6BAFF-R staining on orbital tissues from patients with (A) orbital granulomatous polyangiitis ("GPA"), (B) idiopathic orbital inflammatory disease ("IOID") and (C) orbital sarcoidosis ("S"). (D) Scatter plot showing the mean count of BAFF-R for each of the three orbital diseases.

TABLES

Table 1: Cellular density in histological specimens from patients with GPA and non-GPA (the cell/tissue count was performed in 20 fields of 1mm^2 frame in each slide(total of 20mm^2 in each slide))

Cell & tissue changes	GPA (n=39) Mean ± SE	Non-GPA (n=200) Mean ± SE	Unadjusted	Adjusted	
			p	OR	*p*
Neutrophils	37.2 ± 10.4	11.1 ± 2.4	<0.001 *	0.99 (0.97 - 1.01)	0.26
Eosinophils	50.0 ± 19.4	12.4 ± 2.4	0.002 *	1.00 (0.99 - 1.01)	0.50
Lymphocytes	3292 ± 141	3672 ± 188	0.582		
Macrophages	290.5 ± 19.7	188.9 ± 8.8	<0.001 *	1.00 (0.99 - 1.01)	0.34
Plasma cells	81.8 ± 16.5	66.3 ± 6.4	0.146		
Giant cells	0.26 ± 0.90	0.32 ± 0.10	0.066		
Mast cells	0.33 ± 0.20	0.12 ± 0.03	0.096		
Nuclear debris	3.7 ± 1.5	0.96 ± 0.30	0.011 *	1.08 (0.96 - 1.22)	0.19
Granulomas	0.50 ± 0.40	0.55 ± 0.10	0.046 *	0.12 (0.21 - 0.71)	0.02*
Vasculitis	7.1 ± 0.8	2.95 ± 0.2	<0.001 *	1.33 (1.15 - 1.54)	<0.001*
Necrosis	3.0 ± 0.4	0.35 ± 0.75	<0.001 *	2.39 (1.73 - 3.30)	<0.001*
Fibrosis	6.6 ± 0.5	7.6 ± 0.5	0.906		

* = $p<0.05$

Table 2: Quantitative IHC cell count and comparison between orbital GPA (GPA), IIOD and orbital sarcoidosis (S) (the cell count was performed in 50 fields of 1mm^2 frames in each slide (total of 50mm^2 in each slide))

Cellular marker	Orbital GPA [mean ±se (total)]	IIOD [mean ±se (total)]	Orbital sarcoidosis [mean ±se (total)]	*p* (Kruskal-Wallis)	*p* Post hoc multiple comparison
CD3	5221 ±449 (130532)	3633 ±560 (90827)	4951 ±851 (64369)	0.03*	GPA vs IIOD : p= 0.01* GPA vs S : p= 0.5
CD4	1157 ±145 (28914)	864 ±162 (21606)	955 ±198 (12422)	0.21	
CD8	1041 ±140 (26021)	764.5 ±15.1 (19113)	1015 ±267 (13200)	0.12	
CD68	2202 ±216 (55062)	1152 ±152 (28802)	1561 ±300 (20228)	0.013*	GPA vs IIOD : p<0.001* GPA vs S : p = 0.1

* = p<0.05

Table 3: Comparison of cytokine or receptors markers in tissues from patients with localised granulomatous polyangiitis ("orbital GPA"), idiopathic orbital inflammatory disease ("IIOD), and orbital sarcoidosis; cell counts performed in 50 fields of 1mm^2 frames in each slide (total of 50mm^2 in each slide)

Cell marker	Orbital GPA [mean ±se (total)]	IIOD [mean ±se (total)]	Orbital sarcoidosis [mean ±se (total)]	*p* (Kruskal-Wallis)	*p* (odds ratio) Post hoc multiple comparison
IL-17	270 ±39 (6758)	88 ±9 (2212)	71 ±16 (924)	0.002*	GPA vs IIOD : *p* <0.001 *(high)* GPA vs S : *p* <0.001 *(high)*
IL-23	3186 ±338 (79653)	1461 ±208 (36529)	1461 ±326 (18991)	<0.0001*	GPA vs IIOD : *p*< 0.001 (9) GPA vs S : *p* = 0.002 (high)
BAFF-R	486+103 (12635)	203 ±34 (5080)	238 ±135 (3102)	0.008*	GPA vs IIOD : *p* = 0.002 (6) GPA vs S : *p* = 0.005 (6)
CD20	1025 ±232 (25613)	1026 ±363 (25642)	932 ±213 (12117)	0.40	
CD134	94.6 ±18 (2365)	59 ±11 (1475)	150 ±37 (1955)	0.16	

* = p<0.05

Table 4: Antibodies

Markers	Antibody details	Information
CD3	A0452, DAKO, Cambridgeshire, UK	A T-cell co-receptor that non-covalently associates with the T-cell receptor (TCR) and can be used as a specific T-cell marker.
CD4	Clone 4B12; M7310, DAKO, Cambridgeshire, UK	A surface protein expressed on mature Th cells, CD4-positive T-cells being considered to have a T-helper cell function.
CD8	Clone C8/144B; M7103, DAKO, Cambridgeshire, UK	A co-receptor that is predominantly expressed on the surface of cytotoxic T-cells, and an also be found on natural killer cells, cortical thymocytes and dendritic cells.
CD134	Clone ACT35; 555836, BD Pharmingen™,Becton, Dickinson and Company, Oxford, UK	Also known as OX40, is a member of the TNFR-superfamily of receptors.
CD68	Clone PG-M1; M0876, DAKO, Cambridgeshire, UK	A glycoprotein used as a marker for the various cells of the macrophage lineage.
CD20	Clone L26; M0755, DAKO, Cambridgeshire, UK	A surface protein expressed on surface of all B-cells thatenables optimal B-cell immune response specifically against T-independent antigens.
BAFF-R	11C1; ab16232Abcam, Cambridge, UK	A receptor expressed on B-lymphocytes which selectively binds cytokine B-cell activating factor (BAFF); a potent B-cell activator and plays a role in the proliferation and differentiation of B-cells.
IL-17	IL-17A; AHP455G AbDSerotec, Kidlington, UK	A pro-inflammatory cytokine produced by Th17 (a subset of T-helper cells) that has an important role in inducing and mediating pro-inflammatory responses.
IL-23	H-113; sc-50303, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA	A cytokine produced by macrophages and dendritic cells and influences the development of Th17 and stimulates production of other pro-inflammatory molecules (including IL-1, IL-6, and TNF).

Letter to the Editor

Prevalence and causes of phthisis bulbi in a uveitis clinic

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Editor,

Phthisis bulbi occurs as the end-stage of severe ocular disease and describes a soft collapsed globe con- taining atrophic and disorganised intraocular structures. There is little in the literature pertaining to the devel-opment of phthisis following ocular inflammation (Saeed et al. 2006; Setlur et al. 2010), so we performed a cross-sectional study of 4,000 patients attending a uveitis clinic at Moorfields Eye Hospital to identify patients with severe visual loss and phthisis. Severe visual loss was defined as a best-corrected visual acuity of 20/200 or worse (Jabs et al. 2005), and ocular phthisis was defined as a soft and shrunken globe with evidence of structural disor- ganisation. A total of 333 patients were identified with severe visual loss, indicating a prevalence of 8.3%. Sixty-four of these patients (65 eyes) were diagnosed with phthisis (19%). 51/65 eyes had no perception of light (78%), 10 eyes had perception of light (15%), and four eyes had hand movements vision (6%). 27/64 patients (42%) had visual loss in the other eye, of whom half had severe visual loss (SVL), rendering them legally blind. The mean age at onset of phthisis was 54 years (range 17–97 years) with a Male/Female ratio of 1.3:1. The causes of phthisis are indicated in Table 1. The most com-mon cause was non-infective uveitis, followed by trauma, ocular infection and ocular surgery. Phthisis occurred at mean 2.9 years (range 0–23) after the initiating event, but patients developing phthisis from uveitis took significantly longer to do so at 6.4 ± 7.2 years (mean ± SEM), compared with trauma (1.4 ± 4.3 years) and infection (0.9 ± 1.2 years) ($p = 0.03$, Fisher's exact test). 11/64 patients (17%) were diagnosed with sympathetic ophthalmia. This followed a penetrating eye injury to the other eye in 8/11 patients (73%), of whom seven had had an early enucleation; it followed retinal detachment surgery in the other three patients (27%). 4/11 patients (36%) with sympathetic ophthalmia suffered visual loss, and 3/11 (27%) had SVL; none of the exciting eyes maintained vision. Uveitis was the main cause of phthisis in our study, accounting for 28% of cases, closely followed by trauma and infection. The interval between the time of diagnosis and the development of phthisis was the longest in the uveitis group. Sympathetic ophthalmia developed in 17% of our patients with phthisis, but 7/8 patients in whom it followed a penetrating injury had had an early enucleation, supporting recent evidence that prophylactic enucleation does not prevent the development of sympathetic ophthalmia (Kilmartin et al. 2000; Zhang et al. 2009). Encourag-ingly, most patients retained good functional acuity, 64% maintaining visual acuity of 20/40 or better, and 73% maintaining visual acuity of bet-ter than 20/200.

Table 1. Causes of phthisis in a tertiary referral uveitis clinic.

Category details	Number of patients (%)
Inflammation	18(28)
Panuveitis	14(22)
Chronic anterior uveitis (including JIA)	2(3)
Acute anterior uveitis with iris bombe	1(2)
Sclerokeratitis, Wegener's granulomatosis	1(2)
Trauma	17(26)
Penetrating trauma	11(17)
Blunt trauma	6(9)
Infection	15(23)
Endophthalmitis (postoperative)	5(8)
Endophthalmitis (post-trauma)	2(3)
Endophthalmitis (endogenous)	1(2)
Keratitis with perforation	3(5)
Acute retinal necrosis	4(6)
Postsurgical	6(9)
Multiple retinal detachment surgeries	4(6)
Congenital cataract surgery	2(3)
Miscellaneous	8(12)
Chronic retinal detachment	3(5)
Congenital lesions	2(3)
Neovascular glaucoma	2(3)
Orbital vasculitis	1(2)

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Histopathological features of orbital biopsies associated with a clinical diagnosis of Wegener's granulomatosis

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Introduction

Wegener's granulomatosis (WG) is a presumed autoimmune disease characterised by granulomatous inflammation, small vessel vasculitis and necrotising glomerulonephritis.

Limited WG primarily involves the head and neck region, including the orbit, and is often difficult to diagnose, owing to the lack of classical features of WG and anti-neutrophil cytoplasm antibodies (ANCA).

The purpose of this study was to examine orbital biopsy tissue and determine whether particular histological features were independently predictive of a clinical diagnosis of WG over the subsequent two years.

Methods

This was a retrospective study of orbital biopsies of orbital inflammatory disease undertaken using the UCL Institute of Ophthalmology Pathology Department database. Ethics approval was obtained from the Moorfield & Whittington Research Ethics Committee (REC ref. no. 09/H0721/75, LIGS 1023).

243 biopsies were identified over a period of 15 years. These were analysed and graded in terms of type and degree of cellular infiltration and the presence of granulomas, vasculitis and 'nuclear dust'.

The medical records were identified for each patient, and the clinical history examined to see if a diagnosis of Wegener's Granulomatosis could be made overa subsequent period of at least two years.

Two clinical features were required for the diagnosis to be made, including characteristic ocular involvement, characteristic ENT involvement, renal or pulmonary involvement, a positive immunofluorescence for ANCA or a positive ELISA for anti-PR3 antibodies. Histological findings were excluded from the list of diagnostic characteristics to avoid confounding the results.

Univariate and multivariate analyses were then performed to see which histological features on orbital biopsy were associated with a clinical diagnosis of Wegener's granulomatosis.

Results

Demographics

Of the 234 patients, 43 (18%) were diagnosed with WG and 191 (82%) were diagnosed with other forms or orbital inflammation. 24 patients had WG newly diagnosed based on these histopathology findings, although we required additional clinical criteria for this study (see above). Of the 43 patients with WG, 41 had localised WG and 2 had systemic WG.

Both groups were demographically similar — the mean age was 50 years in both groups and both groups showed a female preponderance (F:M=3:2). 45% of biopsies were from orbital masses and 37% were from the lacrimal gland.

Results

Histopathology:

Histopathological analysis demonstrated a range of acute and chronic inflammatory pictures in both the WG and non-WG groups. Biopsies from patients with a clinical diagnosis of Wegener's granulomatosis had more cellular activity than those from patients without a diagnosis of WG. Granulomas were seen more frequently in patients diagnosed with WG who are ANCA-positive (50%) compared to those who were ANCA-negative (22%)

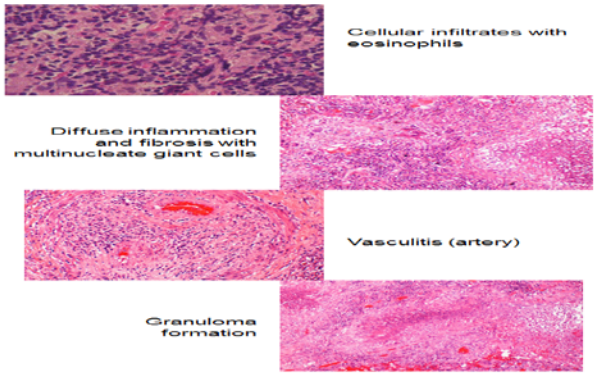
Univariate analysis:

The presence of neutrophils ($p=<0.001$), eosinophils ($p<0.001$), vasculitis ($p<0.001$), histiocytes ($p=0.04$) and nuclear dust ($p=0.03$) within a biopsy specimen were significantly associated with a clinical diagnosis of WG.

Multivariate analysis:

In the multivariate analysis, only neutrophils (OR=3.6,p=0.01), histiocytes (OR=2.9,p=0.02) and vasculitis (OR=2.6, p=0.02) were independently associated with the clinical diagnosis of WG.

Representative histopathology:



Conclusion

These results indicated that there is a significant difference in the levels of cellular activity present in orbital biopsies taken from patients with WG and patients with diagnoses other than WG. In addition, the presence of neutrophils, histiocytes and vasculitis within an orbital biopsy is highly suggestive that the patient will go on to develop clinical features compatible with a diagnosis of orbital WG within two years.

Interestingly, and in contrast to previous reports, eosinophils and necrosis were not significantly associated with a clinical diagnosis of WG. However, there does appear to be an association between granuloma formation within orbital tissue and the production of systemic anti-neutrophil cytoplasm antibodies.

Taken together, these results suggest that analysis of cellular infiltrates may be helpful in diagnosing diseases that are currently often difficult to identify, such as orbital Wegener's granulomatosis. However, diagnosis is still difficult, classic histological features may be absent, and the identification of cellular biomarkers indicative of early Wegener's granulomatosis is still required. Further detailed analysis of the phenotype of infiltrating cells may provide this information, and is currently being studied in our laboratory.

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Acknowledgements

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Analysis of Cellular Infiltration in Orbital Biopsies Of Wegener's and non-Wegener's patients.

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Introduction

Wegener's granulomatosis (WG) is a disease of presumed autoimmune origin that was originally described as characterised by a necrotising granulomatous inflammation of the upper and lower respiratory tract, vasculitis involving small vessels, and a focal necrotising glomerulonephritis.

A limited form of the disease, primarily involving respiratory tract or head and neck region including the orbit, often poses a diagnostic challenge[1], as patients do not develop the classic features of Wegener's[2].

The lack of sensitivity of cytoplasmic antineutrophil antibodies (c-ANCA) makes the initial diagnosis difficult, as it is positive in only 32% of limited Wegener's granulomatosis[3].

Histopathology plays an important role in the diagnosis of the different entities of orbital inflammatory diseases, including orbital Wegener's.

The purpose of our study is to identify the cellular infiltrates in Wegener's granulomatosis and compare their features with those in non-Wegener's granulomatosis.

Methods

A retrospective study of orbital biopsies with a diagnosis of OID was undertaken using the Institute of Ophthalmology Pathology Department database.

160 biopsies with complete histological grading were identified over a period of 15 years and features of biopsies identified as Wegener's granulomatosis were compared to those of other diagnoses.

Results

The spectrum of diagnoses made included chronic idiopathic inflammation of the orbit (42%), lymphoid hyperplasia (17%), Wegener's granulomatosis (16%,) dacroadenitis (9%), sarcoidosis (3%), eosinophilic angiocentric fibrosis (2%), myositis (2%), lymphoma (1%) and others (8%).

Of the 160 biopsies, fibrosis was the most frequent feature seen, being present in 77% of biopsies. Other histological features identified include granuloma 36%, follicles 34%, vasculitis 20% and necrosis 23%

Cellular infiltrates that characterised orbital inflammation are eosinophils 64%, lymphocytes 45%, polymorphonucleocytes (PMNs) 36%, plasma cells 35% and mononuclear granulocytes (MNGs) 18%.

Results

Of the 25 biopsies that were recognised as Wegener's granulomatosis had the most cellular activity, with eosinophils being present in 100% of biopsies, PMNs in 92%, lymphocytes in 48%, MNGs in 36%, plasma cells in 32%.

Vasculitis was present in 64%, necrosis 68% and granuloma 76% in biopsies suggestive of Wegener's granulomatosis. The classic triad of vasculitis, necrosis and granulomatous inflammation was rarely seen, present in only 36%. Other features seen included fibrosis in 88%, and sclerosis in12%.

In the non-Wegener's group, cellular infiltrate comprised of eosinophils (58%), lymphocytes (44%), plasma cells (36%), PMNs (25%) and MNGs (15%).

Vasculitis, necrosis and granulomatous inflammation was seen in 12%, 15% and 29% of orbital biopsies respectively.

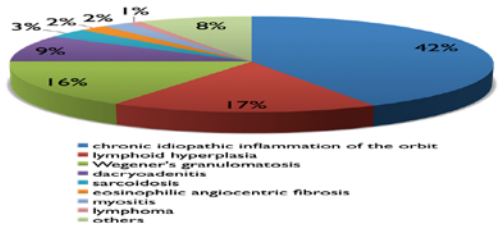

Figure 1. Spectrum of histopathological diagnosis

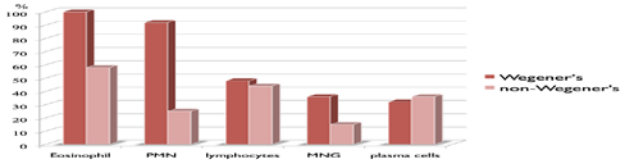

Figure 2. Comparison of cellular infiltrates in Wegener's and non-Wegener's biopsies

Results

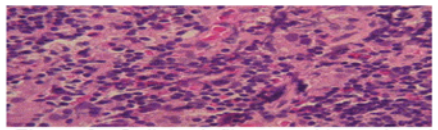

Figure 3. Cellular infiltrates with eosinophils

Discussion

Our series demonstrates that Wegener's granulomatosis has non-specific histopathological features with the classical triad of vasculitis, necrosis and granulomatous inflammation present in only 36%. This is lower than in previous studies showing the classic features in 50% of patients[4,5].

In all the biopsies with histological features consistent with Wegener's granulomatosis, eosinophils are present in variable numbers. This is an interesting finding in the light of studies suggesting that eosinophils may play a role in disease progression in Wegener's granulomatosis[6].

However, further studies need to be conducted to ascertain the clinical significance of eosinophils in our patients.

Conclusions

Analysis of cellular infiltrates may be helpful in diagnosing diseases that are currently often difficult to identify, such as orbital Wegener's granulomatosis.

Classic histological features may be absent and earlier cellular biomarkers indicative of Wegener's granulomatosis are needed.

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Wegener's granulomatosis
Radiological features of lacrimal gland involvement.

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Introduction

Lacrimal gland involvement in Wegener's granulomatosis (WG) has been well described and may be the initial presenting feature preceding systemic symptoms[1-4]. WG can have a wide range imaging characteristic[5] making it difficult to distinguish from other orbital inflammatory conditions, especially if lacrimal gland enlargement is the chief presentation.

The purpose of the study is to describe the imaging findings of various pathologies in the lacrimal fossa and compare the radiological features of patients presenting with lacrimal gland enlargement secondary to WG with those of other orbital inflammatory diseases.

Methods

Ethical approval for the study was granted by the Moorfields and Whittington Research Ethics Committee (LIGS 1023).

A retrospective analysis of all patients undergoing orbital biopsy from 1988-2006 was undertaken and clinical details obtained from the case notes. Imaging details were obtained from the radiology reports and available imaging studies were viewed in hard copy or using medical diagnostic grade PACS viewing systems.

Results

85 patients with predominantly lacrimal gland enlargement at presentation were included in the study. Their mean age was 46.7 years old (median 47, range 12-83) and male:female ratio was 1:2.

The main causes of lacrimal gland enlargement were non-specific dacryoadenitis (34%), idiopathic orbital inflammatory disease (IOID) (34%), sarcoidosis (14%), WG (8%), lymphoid hyperplasia (7%), lymphoma (4%) and a miscellaneous group (10%).

The most frequent radiological manifestations were orbital mass in 52% and diffuse inflammation in 46%. Other features included extraocular muscle involvement (33%), infiltration of orbital fat (18%), optic nerve involvement, sinonasal involvement (11% respectively), bony changes (5%) and intracranial extension (4%). Imaging features for individual conditions were summarised in tables 1 and 2.

Results

Imaging features	WG	Dacryoadenitis	Sarcoidosis	IOID
Associated mass or infiltration	57%	14%	15%	85%
Muscle cone involvement	29%	7%	15%	75%
Sinonasal involvement	29%	3%	8%	5.0%
Bony changes	14%	0	8%	5.0%
Optic nerve involvement	14%	0	8%	30%
Intracranial extension	0	0	0	15%

Table 1. Details of imaging features in orbital inflammatory conditions with lacrimal gland enlargement.

Imaging features	WG	Dacryoadenitis	Sarcoidosis
Diffuse enlargement	100%	62%	50%
Well-defined margins	57%	50%	80%

Table 2. Specific imaging features of the lacrimal gland in inflammatory conditions with predominantly lacrimal gland involvement.

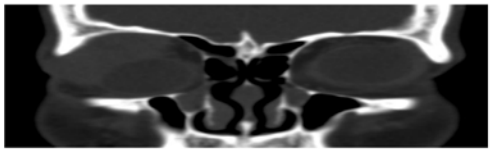

Figure 1. Coronal CT image demonstrate bony scalloping of the right orbital roof adjacent to a large mass arising in lacrimal fossa causing proptosis and marked displacement of the globe inferonasally.

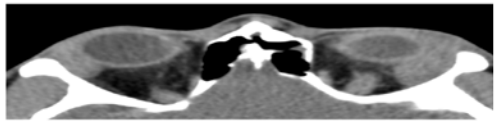

Figure 3. Axial CT image demonstrate bilateral asymmetrical enlargement of the lacrimal gland with involvement of both the orbital and palpebral portions.

Results

A higher proportion of patients with WG demonstrated sinonasal and bony involvement *(figure 1)* compared dacryoadenitis, sarcoidosis and IOID (OR = 11.2, 4.8, 7.6 for sinonasal involvement; OR = ∞ , 2.0, 3.1 for bony changes respectively).

There is a trend towards diffuse involvement of both the palpebral and orbital lobes in patients with WG *(figure 2)*.

Discussion

Sinonasal involvement is common in WG and has been reported in 69% of patients with orbital and adnexal WG[6]. A range of bony changes in WG have been described including bony erosion, scalloping, sclerosis and calcification of the orbit, paranasal sinuses and greater wing of sphenoid[5,7].

In our study, opacification of the sinuses, inflammatory mucosal changes and bony scalloping adjacent to the lacrimal gland mass were demonstrated, with one patient eventually progressing to extensive sinonasal disease and collapse of nose bridge.

Lacrimal gland enlargement may be the initial presentation of aggressive orbital and adnexal WG, with some critical features present only on subsequent imaging.

Conclusions

Associated sinonasal involvement and bony changes on imaging are highly suggestive of WG and should prompt a full diagnostic workup. Sequential imaging is useful to assess the progression of disease and detect diagnostic features which may not be evident at presentation.

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Phthisis bulbi in patients with ocular inflammation

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Introduction

Uveitis is an important cause of visual loss, especially in the working population[1] and accounts for 3-10% of visual loss in various parts of the world [2, 3].

Severe end-stage inflammation may result in phthisis bulbi – a soft atrophic eye with structural disorganisation.

Phthisis is a major indication for enucleation both in the developing and developed world [4, 5]. In the United Kingdom, phthisis is the second commonest indication for enucleation or evisceration in the last decade after ocular trauma [6], and has consistently accounted for approximately 10% of enucleations over the past 60 years [7].

The aim of this study was to determine the prevalence and main causes of phthisis in patients attending a uveitis clinic in a tertiary referral centre, and to identify any factors associated with its

Methods

Ethical approval was obtained from the Research Governance Committee of Moorfields Eye Hospital (protocol LIGS1021). We performed a cross-sectional case-notes study of 2,500 patients attending a uveitis clinic at Moorfields Eye Hospital.

Ocular phthisis was defined as a soft shrunken globe on clinical examination with evidence of structural disorganisation (Fig 1).

Fig 1. Phthisis bulbi - a soft atrophic eye.

Results

We identified 64 patients with 65 phthisical eyes, indicating a prevalence of 2.6% within the clinic. The most common cause of phthisis was non-infective uveitis, accounting for 18 patients (28%). Trauma was responsible in 17 patients (27%), ocular infection in 15 patients (23%) and ocular surgery in six patients (9%). Other less frequent causes included chronic retinal detachment, neovascular glaucoma and orbital vasculitis (Table 1).

Category	Details	Number of patients
Inflammation (28%)	Panuveitis	14
	Chronic anterior uveitis, JIA	2
	Acute anterior uveitis with iris bombe	1
	Sclerokeratitis, Wegener's granulomatosis	1
Trauma (27%)	Penetrating trauma	11
	Blunt trauma	6
Infection (23%)	Endophthalmitis (post operative)	5
	Endophthalmitis (post trauma)	2
	Endophthalmitis (endogenous)	1
	Keratitis with perforation	3
	Acute retinal necrosis	4
Post surgical (9%)	Multiple retinal detachment surgery	4
	Congenital cataract surgery	2
Miscellaneous (13%)	Chronic retinal detachment	3
	Congenital lesions	2
	Rubeotic glaucoma	2
	Orbital vasculitis	1

Table 1. Causes of ocular phthisis

Phthisis occurred a mean of 6.4 years (range 0-23 years) after the diagnosis of uveitis compared to 1.5 years (range 0-17 years) for phthisis from other causes (Table 2) ($p=0.03$).

Category	Mean time to phthisis (± SD) in years	Range (years)	% developing phthisis within 2 years
Inflammatory	6.4 (± 7.2)	0-23	88
Surgery	2.2 (±5.3)	0-13	83
Trauma	1.4 (±4.3)	0-17	81
Infection	0.9 (±1.2)	0-3	35

Table 2. Duration to development of phthisis

Results

51/65 eyes were NPL. 13 of these had been eviscerated or enucleated, with the majority of enucleations following penetrating eye injuries (65%) or infection (15%). A further ten eyes were PL, and three eyes had hand movements vision (Fig 3).

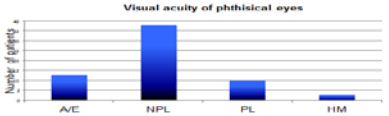

Figure 2. Visual acuity of phthisical eyes

27 patients (42%) had visual loss in the other eye, of whom 14 patients (22%) had severe visual loss, rendering them legally blind (Fig 4). The most common cause of severe visual loss in the contralateral eye was ocular inflammation, including three cases (21%) of sympathetic ophthalmia (SO).

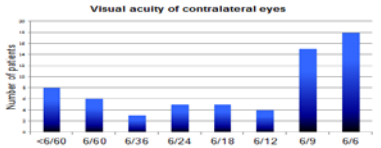

Figure 3. Visual acuity of contralateral eyes

11 patients were diagnosed with SO. In eight cases (73%), this followed a penetrating eye injury to the other eye, which had led to an enucleation in seven patients. SO followed multiple surgeries for retinal detachment in the other three patients.

Four patients (36%) with SO suffered visual loss, of whom three patients (27%) had severe visual loss. None of the exciting eyes maintained vision, and 55% of them had been enucleated, all within two years of the causative event.

Conclusions

Uveitis was the main cause of phthisis in our study, accounting for 28% of cases, closely followed by trauma and infection. Panuveitis accounted for over three-quarters of phthisis in the uveitis group, which is consistent with previous studies showing that panuveitis has the worst visual prognosis among the uveitides, causing over 40% of visual loss [1].

The interval between the time of diagnosis and the development of phthisis was the longest in the uveitis group and was also the most variable, averaging 6.4 years and ranging from several months to 23 years, probably due to variability in the rate of development of ciliary body shutdown and hypotony as a result of cumulative damage from recurrent cycles of inflammation.

In contrast, the average time taken to develop phthisis is shorter in trauma and infection, and a much higher proportion of patients developed phthisis within 2 years. Mechanical disruption, including the loss of intraocular contents in penetrating trauma and perforated infective keratitis, may be contributory factors in this rapid development of phthisis.

Uveitis is an important cause of phthisis, and the interval to phthisis is significantly longer compared to phthisis from other causes. 42% of patients with phthisis had visual loss in the other eye, with 22% being legally blind.

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No.	study number	Age	Gender	known WG	ANCA presentation	ANCA later	ANCA positive	ANCA comments	diagnosis WG	**Clinical dx comments**
1	2	38	F	N	Y	Y	Y	PR3+ve	1995	orbital WG with systemic features
2	12	73	F	N	N	N	N		1994	localised WG, max sinus granuloma 92
3	14	64	F	N	N	N	N		1998	WG, op notes only, DCR, no other info
4	26	52	M	N	N	N	N		2006	gran inflam?limited/orbital WG
5	33	83	F	Y	U	U	N		2001	known WG, no details
6	50	28	M	Y	Y	N	Y		1983	known WG nasal, orbital, for bilat DCR
7	74	39	M	Y	N	N	N		2003	WG 2003 sinus, middle ear, hearing loss mucocoele
8	87	25	M	N	N	N	N		2002	orbital and sinus wg
9	90	53	M	N	N	N	N		2005	poor vision since 1996, prev dx as neurosarcoid > bx brain, no sarcoid, be orbital mass
10	91	86	M	N	Y	U	Y		1998	orbital WG
11	124	46	M	Y	N	N	N		1989	known localised WG 89, bilat blocked NLD
12	133	31	M	Y	Y	Y	Y		1991	orbital WG, known systemic aortic, on Cyclophos 92
13	135	57	M	Y	N	N	Y		2003	WG, nasal, lung, kidney. Pp. Op notes only DCR
14	147	14	F	N	Y	Y	Y	c-anca +ve	1999	Orbital WG
15	192	36	M	Y	Y	Y	Y		1998	orbital WG
16	240	69	F	Y	N	U	Y		2003	known WG diagnosed at another hospital, pulmonary fibrosis, ent (bleed, 2 ops), NLD obstn for DCR, ant uveitis and cmo post cat
17	255	78	F	N	N	N	N		1995	orb pseudotumour
18	256	66	F	N	N	N	N		2001	orbital WG
19	258	26	F	Y	N	U	N		1995	limited, known WG sinonasal, R NLD obsn, LS abscess
20	272	71	F	N	Y	Y	Y		1998	orbital WG
21	291	35	F	Y	U	N	N		1999	limited WG sinus orbits. TED for decomp
22	294	35	M	N	N	U	N		2009	cxr and kindney normal
23	301	67	M	Y	N	N	Y		2002	sinonasal WG
24	303	57	F	N	Y	U	Y		2006	ANCA raised in Hammesmith once
25	305	30	M	N	Y	Y	Y		2007	limited orbital WG
26	307	35	F	Y	N	U	Y		2008	known wg with nld block
27	311	42	F	Y	Y	Y	Y	c anca	2005	limited wg, blocked nld, scleritis march 2005, dx wg oct 2005, collapse bridge 2006, epiphora 2008 > leaking left sinus
28	317	54	M	N	N	Y	Y		2008	orbital WG

29	338	27	F	N	N	Y	Y		2007	symptom started 2005, histo syggestive but not confirmed with blood test, anca positive later in 2007,limited orbital WG left eye
30	361	87	F	Y	N	N	N		2005	limited WG - ent, orbit
31	375	56	F	Y	U	U	N		1996	known WG, blocked DCR
32	390	65	F	Y	N	U	Y		1986	known limited WG 1994 on MTX pred, R mucocoele DCR
33	417	50	F	Y	Y	N	Y		2000	WG clinically, limited, facial pain
34	444	35	M	Y	Y	N	Y		2007	known WG aorta, big vessels
35	449	57	F	Y	Y	Y	Y		1995	known systemic WG 1995 on Aza
36	467	57	F	N	N	Y	Y	p-ANCA 160	2006	limited orbital WG

No.	study number	Tissue	combi tissues	orbit	LG presentn	Nld	Muscle/eyelid/conj	ent	lungs	heart	kidney	cns	progress
1	2	O		Y	N	N	N	Y	N	N	Y	N	N
2	12	COMBI	O+L	Y	Y	N	N	Y	N	N	N	N	N
3	14	O		Y	N	N	N	Y	N	N	N	N	N
4	26	O		Y	N	N	N	N	N	N	N	N	N
5	33	COMBI		N	N	Y	N	Y	N	N	N	N	N
6	50	COMBI	N+Ls	N	N	Y	N	Y	Y	N	N	N	N
7	74	COMBI	Ls+N	N	N	Y	N	Y	N	N	N	N	N
8	87	LG		N	Y	N	N	N	N	N	N	N	N
9	90	O		Y	N	N	N	Y	N	N	N	N	N
10	91	O		Y	N	N	N	N	N	N	N	N	N
11	124	COMBI	Ls+N	N	N	Y	N	Y	N	N	N	N	N
12	133	COMBI	O+L+obicularis	Y	Y	N	Y	Y	Y	Y	N	N	N
13	135	COMBI	Ls+N	N	N	Y	N	Y	Y	N	Y	N	N
14	147	O		Y	Y	N	N	Y	N	N	N	Y	N
15	192	O		Y	N	N	N	Y	N	N	N	N	N
16	240	COMBI	Ls+O	Y	N	N	N	Y	Y	N	N	N	N
17	255	O		Y	N	N	N	Y	N	N	N	N	N
18	256	N	nasal septum	N	N	Y	N	Y	N	N	N	N	N
19	258	COMBI	N+Ls	N	N	Y	N	Y	N	N	N	N	N
20	272	COMBI	enucleation	Y	N	Y	Y	Y	N	N	N	N	N
21	291	O		Y	N	N	N	Y	N	N	N	N	N
22	294	O		Y	N	N	N	Y	N	N	N	N	N
23	301	COMBI	Ls+N	N	N	Y	N	Y	Y	N	N	N	N
24	303	COMBI	O+L	Y	Y	N	N	Y	N	N	N	N	N
25	305	O		Y	Y	N	N	N	N	N	N	N	N
26	307	COMBI	Ls+N	N	N	Y	N	Y	Y	N	N	N	N
27	311	COMBI	Ls+N	N	N	Y	N	Y	N	N	N	N	N
28	317	COMBI	L+O	Y	Y	N	N	Y	Y	N	N	N	N

29	338	O		Y	Y	N	N	N	N	N	N	N	N
30	361	COMBI		Y	N	N	Y	Y	N	N	N	N	N
31	375	COMBI	Ls+N+ethmiod	N	N	Y	N	Y	N	N	N	N	N
32	390	COMBI	Ls+N	N	N	Y	N	Y	N	N	N	N	N
33	417	LG		N	Y	N	N	Y	N	N	N	N	N
34	444	COMBI	O+Lid+skin	Y	N	N	Y	Y	Y	Y	N	N	N
35	449	LG		N	Y	N	N	Y	Y	N	N	N	N
36	467	COMBI	LG+O	Y	Y	N	N	N	N	N	N	N	N

No.	study number	**main eye symptom**	Laterality	VA R	VA L	final VA R	final VA L	VA improve	decreased VA	decreased colour	pain	proptosis	diplopia
1	2	proptosis diplopia	R	6/5	6/5	6/4	6/4	S	Y	N	N	Y	Y
2	12	orbital swelling	R	6/18	6/12	NPL	6/9	N	N	N	Y	Y	Y
3	14	blocked nld	L					S	N	N	N	N	N
4	26	lower lid swelling	L	6/9	6/9	6/9	6/9	S	N	N	N	N	N
5	33	nld block > dcr	R	6/9	PL	6/12	PL	S	N	N	Y	N	N
6	50	nld block > dcr	L	6/6	6/5	6/6	6/12	N	N	N	N	N	N
7	74	nld obstruction, mucocele	R	6/6	6/6	6/5	6/6	S	N	N	N	N	N
8	87	ul ptosis with mass	R	6/5	6/5	6/36	6/5	N	Y	N	Y	N	N
9	90	poor vision and diplopia	B					S	N	N	N	N	N
10	91	lower lid swelling	R	6/9	NPL	6/12	NPL	N	Y	N	N	Y	N
11	124	blocked nld	R	6/6	6/12	6/5	6/9	S	N	N	N	Y	Y
12	133	swelling and diplopia	R	6/12	6/6	6/12	6/6	S	N	N	N	Y	Y
13	135	nld block > dcr	B					N	N	N	N	N	N
14	147	ptosis, hypoglobus	L	6/5	6/12	NA		S	Y	N	N	Y	N
15	192	EOM limitation, previous scleritis, prev DCR RL	R	6/5	6/5	6/4	6/4	S	N	N	N	Y	Y
16	240	epiphora/nld block/dacryocystitis	L	6/12	6/9	6/60	6/36	N	N	N	N	N	N
17	255	frozen orbit 1 year later despite steroids, sinusitis ?WG	L	6/9	6/9	6/9	6/9	S	N	N	Y	Y	Y
18	256	left ptosis and diplopia	L	6/5	6/6	6/4	6/5	Y	N	N	N	N	Y
19	258	dacryocystitis > dcr	R	6/5	6/5	6/5	6/5	S	N	N	N	N	N
20	272	scleritis	R	PL	6/5	A/E	6/4	N	Y	Y	Y	N	N
21	291	epiphora/nld block/dacryocystitis	R	6/5	6/4	6/4	stable Feb 10	S	N	N	Y	Y	Y
22	294	left proptosis and reduced vision with redness	L	6/5	HM	6/5	CF	S	Y	Y	Y	Y	Y
23	301	nld block > dcr	L	6/5-2	6/5-2	6/6		S	N	N	N	N	N
24	303	left periorbital swelling	L	6/6	6/36	6/5	6/12, 6/6	Y	Y	N	Y	Y	Y
25	305	left lac gland enlargement	L	6/6	6/6	6/4	6/5	S	N	N	Y	N	N
26	307	nld block > dcr	L	6/5	6/5	6/5	good	S	N	N	N	N	N
27	311	nld block > dcr	L	6/5	6/4	6/5	good	S	N	N	N	N	N

28	317	orbital swellinh	R	6/9	6/5	6/6	6/4	Y	Y	N	N	N	N
29	338	orbital swelling, proptosis and diplopia	L	6/6	6/6			S	N	N	Y	Y	Y
30	361	diplopia and motility limitation, fibrosis, pain	R	6/12	6/12	NPL	not rx old frail, stable	N	N	N	Y	Y	Y
31	375	nld block > dcr	R	6//9	6/6	6/9	disch	S	N	N	N	N	N
32	390		R	6/5	6/4	6/6	improved, disch	Y	N	N	N	N	N
33	417	limitation, lid swelling, diplopia	L	6/6	6/6	6/6	FU Hams, persistent facial pain	S	N	N	Y	N	Y
34	444	painful orbital swelling and then nld block > dcr	L	6/5	6/5			S	N	N	N	Y	Y
35	449	dacryoadenitis	R	6/9	6/6	6/6	no active ds	S	N	N	Y	Y	N
36	467	hypoglobus, scleritis, PUK 2 and 3 y later	L	6/6	6/9	6/9	6/12	S	Y	Y	Y	Y	N

No.	study number	lid swelling	lid redness	red eye	EOM limitation	globe displacement	sinonasal	other signs	Systemic present	Systemic presentation	Systemic later	Systemic details
1	2	Y	N	N	Y	Y	Y	hypoglobus	N	N	N	
2	12	Y	Y	N	Y	Y	Y	epiphora, ptosis, frozen orbit; scleritis, LOV 2y later	N	N	N	unknown
3	14	N	N	N	N	N	N		N	N	N	
4	26	Y	N	Y	N	N	N	indurated mass L LL	N	N	N	
5	33	Y	Y	N	N	N	Y	dacryocystitis	U	U	U	known WG?system on Pred and Aza
6	50	N	N	N	N	N	Y	epiphora 2000, sleritis 2004	N	N	N	
7	74	N	N	N	N	N	Y	R epiphora	N	N	N	
8	87	Y	Y	N	N	N	N	ocular surface	N	N	N	
9	90	N	N	N	N	N	N		U	N	N	
10	91	N	N	N	Y	Y	N	lat globe displcm, optic neuropathy ?compressn	N	N	N	
11	124	N	N	N	Y	N	Y	epiphora bilat	N	N	N	sinonasal, arthritis
12	133	Y	N	Y	Y	N	N	R RAPD, disc swelling, sclerokeratitis, orbital oedema 96	N	Y	Y	limited wg, tracheal, aortic inflm 91 on
13	135	N	N	N	N	N	N		Y	Y	N	lung, kidney, nasal
14	147	N	N	N	Y	Y	N	ptosis, hypotropia	N	N	Y	DI, intracranial invovlem
15	192	Y	Y	N	N	N	N	EOM limitation, previous scleritis, prev DCR RL	N	Y	Y	known limited WG on MMF, MTX, pred
16	240	N	N	N	N	N	Y	epiphora	N	N	N	previous known WG ? Course ?rx
17	255	Y	N	N	Y	N	N	frozen orbit 1 year later despite steroids, sinusitis ?WG	N	N	N	ESR 86
18	256	N	N	N	N	N	N	LL retraction,conj-nasal fistula	N	N	N	
19	258	Y	Y	N	N	N	Y	R periorb cellulitis, LS abscess, epiphora	N	N	N	
20	272	N	N	Y	N	N	N	sclerokeratitis, pannus, AION	N	N	Y	deafness
21	291	Y	N	N	N	N	Y		N	N	N	previous epiphora dx WG
22	294	Y	N	N	Y	Y	N	RAPD	N	N	U	
23	301	N	N	N	N	N	Y	epiphora, dacryocystitis	N	N	N	upper airways, nasal. Middle ear
24	303	Y	Y	N	Y	Y	N	ON, ptosis, hypoglobus, subglottis stenosis	N	N	N	
25	305	Y	N	Y	N	N	N	LG swelling l>R, L conj congestion	N	N	N	
26	307	N	N	N	N	N	Y	epiphora	Y	Y	Y	known wg kidney and lung
27	311	Y	Y	N	N	N	N	epiphora	N	Y	N	known wg, orbit and sinonasal

28	317	Y	N	N	N	N	N	R lid swelling recurrent	N	N	N	
29	338	Y	N	N	N	N	N		N	N	N	
30	361	Y	Y	Y	Y	Y	Y	mucocoele, fixed globe	N	N	N	treated conservative although has orbit age
31	375	N	N	N	N	N	Y	Lepiphora	N	N	N	known WG
32	390	N	N	N	N	N	Y	dacryocystitis sticky discharge	N	N	N	known limited WG
33	417	Y	N	Y	Y	N	Y	nasal crusting, collapse nose bridge 07	N	N	N	
34	444	Y	Y	Y	Y	Y	Y	preseptal cellulitis, mucocoele	N	Y	Y	aorta, cerebral aneurysm
35	449	Y	N	N	N	N	N	LG enlargem, necrotic defect R UL	Y	Y	Y	upper airways
36	467	N	N	N	N	Y	N	hypoglobus, scleritis, PUK 2 and 3 y later	N	N	N	

No.	study number	immunosuppressive	number of immunosuppressives	details	surgery	number of recurrences	No of meds for control	Outcome	Date 1st seen	Date last seen	months
1	2	Y	3	MMF 2000, tacrolimus 96, aza 97	DCR RL	ESRF GN 1975, tx 76, 2000			30/10/1996	26/11/2008	145.01
2	12	Y	1	cyclophos		1	2	Telford 97	14/6/1994	2/7/1997	36.65
3	14								1/11/1995	11/3/1999	40.34
4	26	Y	1	Aza		0	2	mid Essex April07	31/5/2006	4/7/2006	24.00
5	33	Y	1	Aza	DCR, LCS			discharge	23/8/2001	2002	24.00
6	50	Y	4	CPM,MTX,Infix, Ritux	RDCR00, LDCR01, L phaco 06	2		Belfast 07	1/6/2002	31/1/2007	56.09
7	74	Y	1	Aza	R DCR			discharged Dec 06	25/5/2006	21/12/2006	24.00
8	87	Y	2	aza +rtx	lid repair and debulking of lac gland				19/6/2002	11/7/2011	108.82
9	90	Y	1	cyc					5/9/2005	14/9/2005	24.00
10	91	Y	1	cyclophosphamide		1	2	frozen orbit	1/8/1998	2/12/1998	24.00
11	124	Y	1	CPM, Aza	bilat DCR	1	2	?FU	31/3/1994	23/9/1998	53.82
12	133	Y	1	CPM started before				back to Edin, refer Cambridge monoclonal Ab	4/3/1997	17/3/1997	24.00
13	135								20/4/2005	11/8/2009	51.78
14	147	Y	3	MTX, Aza, (CPM initial)			3	FU central middlesex	1998	2003	60.00
15	192	Y	1	MTX		2	2	epiphora, no proptosis	1998	2000	24.00
16	240	N			DCR			recurrent AU, topical drops, cat extn, CMO, disch	15/12/2004	5/3/2009	50.70
17	255					1	1	local FU for frozen orbit severe diplopia	16/3/1995	13/3/2000	60.00
18	256	N					1	DNA FU Devon	24/1/2001	2/4/2003	26.27
19	258	Y	1	Aza, Septrin	DCR	0		Disch	20/12/2001	21/2/2002	24.00
20	272	Y	1	Aza	Enucleation	1	2		22/1/1998	13/11/2002	57.76
21	291	Y	1	MMF, Ritux. Previous Aza toxy	R orb debulk, L lat decomp	2	5/4		1999	2001	24.00
22	294	N						back to Guys vasculitis	30/9/2009	18/11/2009	24.00
23	301	Y	1	MTX	DCR bilat	2	6/9		4/1/2006	21/4/2011	63.58

24	303	Y	1	Aza				11/10/2006	11/10/2006	6/12/2006	24.00
25	305					2		current FU Hams	4/7/2007	3/8/2011	49.05
26	307	Y	1	already on MTX, had rituximab for systemic WG	DCR	2	6/5	20/11/2008	10/1/1997	12/3/2009	146.14
27	311	Y	4	2005 pred and azt, flare up when pred 5, oct 2008 mtx pred + cellcept, iv cyclophosphamide 2009 2 courses, stopped due to vaginal bleed	dcr		6/9		7/5/2009	5/5/2011	23.97
28	317	Y	1	Aza		2		14/3/2008	31/12/2005	10/6/2009	41.36
29	338								25/9/2006	22/6/2011	56.94
30	361	N				0	6/9	29/6/2005	29/6/2005	14/2/2008	31.59
31	375	N			DCR redo	0	6/9	10/4/2008	4/8/2008	2010	24.00
32	390	Y	1	MTX from previous	R DCR	0	6/6	22/6/2006	3/3/1999	27/9/2007	102.93
33	417	Y	2	Aza, MTX		3	6/6	20/12/2006	16/9/2009	2011	24.00
34	444	N							2/2/2008	13/4/2011	38.37
35	449	Y	3	Aza then MTX then RTX	Bilat DCR06	2	6/9	2/5/2008	26/3/2001	21/6/2011	122.95
36	467	Y	2	MMF 06, CPM 09	tectonic graft for PUK 09	1		3/5/2006	2/6/2009		24.00